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14. ABSTRACT
Our proposal is to explore the novel notion that it may be possible to restore near normal HR activity in Brca1 cells and tissues. We believe that this phenomenon will lead to targeted therapies to reduce lifetime risk of tumor formation in BRCA1 and potentially BRCA2 carriers.

15. SUBJECT TERMS
BRCA1, 53BP1, cancer biology, DNA repair, tumorigenesis.

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Table of Contents

Page

Introduction

Genomic instability is a hallmark of cancer. Central to a cells ability to maintain genomic stability are systems that monitor and repair DNA double strand breaks (DSBs). The objective of this study is to understand how the choice of pathways used to repair DNA damage determines whether the repair is error free or causes genomic instability. In mammalian cells, homologous recombination (HR) and non-homologous end joining (NHEJ) are the two major pathways involved in the repair of DNA DSBs. The Brca1 gene is required for DNA repair by homologous recombination and normal embryonic development. The protein 53BP1 promotes ligation and facilitates end joining. In previous studies, we have demonstrated that Brca1 and 53BP1 can compete for the processing of DSBs and that 53BP1 can promote genomic instability in the absence of Brca1. Thus, the tumor suppressive function of Brca1 does not appear to be absolute and can be modulated by altering the ability of cells to carryout NHEJ. Our study focuses on shifting the balance between these two repair pathways (HR and NHEJ) to restore error free repair and genomic stability. We believe that a better understanding of mechanisms of DSB repair pathway choice may have important therapeutic implications for prevention or treatment of Brca1/2 germline mutation-associated cancers.

Body

Aim 1: ***Determine the capacity of NHEJ deficiency to rescue defects in homologous recombination (HR). Using various established mouse models where there is a clearly described defect in HR, we will test the role of NHEJ proteins 53BP1 amongst others in subverting HR.***

- Work initially described in our 2013 annual summary report identified a factor called RIF1 that acts downstream of 53BP1 in blocking resection. This work was recently published in ***Science*** (M. Di Virgilio, 2013). A summary of the relevant data is shown in Fig. 1 below (and in Fig. 5 of the cited ***Science*** paper). Rif1 is a phosphorylation-dependent interactor of 53BP1. In the absence of RIF1, we observe enhanced DNA resection, a reduction in class switch recombination (CSR) and increased DSBs. In **work in progress**, to fully assess the impact of RIF1 in the repair process, we have generated CD19 CRE x BRCA1f/f RIFf/f double knockout B cells, and will examine their sensitivity to PARPi, proliferation capacity and ability to form RAD51 foci. RAD51 catalyzes strand exchange between sister chromatids or homologous chromosomes and its presence would be indicative of error-free repair.

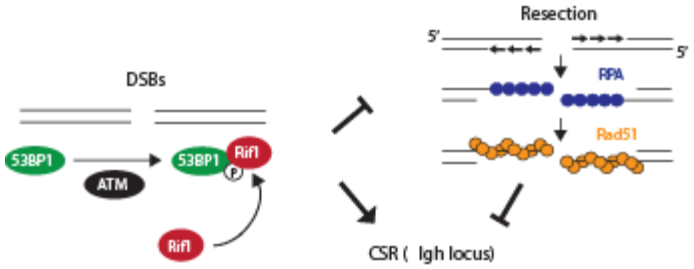

Fig. 1. **DNA damage activates ATM, which phosphorylates multiple targets, including 53BP1. This event recruits Rif1 to 53BP1 at the DSB, where it inhibits DNA resection. The extensive resection in the absence of Rif1 impairs class-switch recombination, for example at the Igh locus.**

- PTIP is a multifunctional protein that, like RIF1, interacts with phosphorylated 53BP1. Ablation of PTIP phenocopies the 53BP1 deletion in that it promotes genome stability and survival in BRCA1 mutant B cells. This work has been described in a recent paper in ***Cell*** (Fig. 5 of the cited ***Cell*** paper, Callen, 2013) and in Fig. 2 below. Loss of PTIP increased HR in BRCA1 mutant cells by promoting DSB

resection, increasing Rad51 foci formation and decreasing sensitivity to DNA damaging agents such as PARP inhibitors (PARPi) (Fig.2).

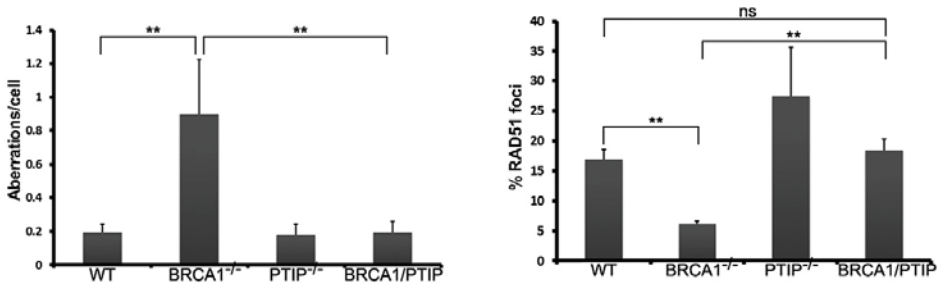

Fig. 2. **Ablation of PTIP rescues homologous recombination in BRCA1-deficient cells, as measured by reduced genome instability in the presence of PARPi (left panel) and increased Rad51 foci formation (right panel).**

In **work in progress**, we have examined the sensitivity of BRCA1/PTIP deficient cells to cisplatin. While BRCA1/53BP1 deficient cells are sensitive to cisplatin (described in our previous annual report and in Bunting et al., 2012), we have found that BRCA1/PTIP-mutant cells are resistant as measured by metaphase analysis (Fig. 3A). While BRCA1 is implicated in an upstream role in homology-directed repair, which is counteracted by 53BP1, BRCA2 functions later by promoting RAD51 filament formation. We have generated BRCA2/PTIP-doubly deficient B cells (CD19 CRE BRCA2f/fPTIPf/f) and measured their sensitivity to PARPi and cisplatin. Strikingly, in contrast to BRCA2/53BP1 mutants, which are hypersensitive to both DNA damaging agents, BRCA2/PTIP deficient cells are resistant (Fig. 3B). Based on these results, we hypothesize that in addition to its role in modulating 53BP1-dependent DSB resection, PTIP could also function independently of 53BP1 in regulating DNA repair choice.

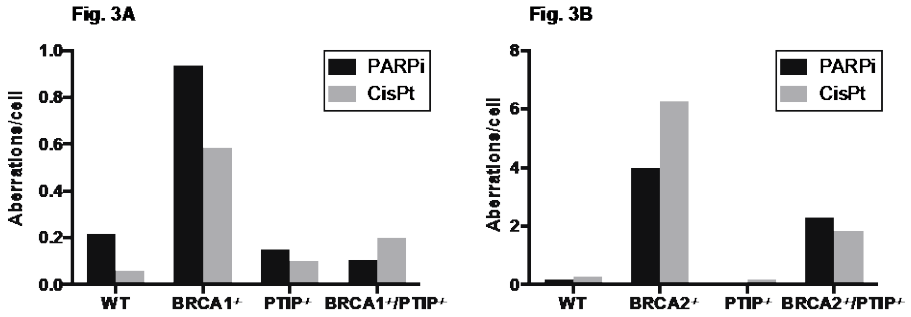

Fig. 3. PTIP ablation confers PARPi and cis-platin resistance in BRCA1/2-deficient cells.

Aim 2: ***Determine the domain of 53BP1 that inhibits HR in Brca1-deficient mice. We will use a combined in vitro and in vivo reconstitution approach to define the functional domains of 53BP1 that regulate the observed HR defects seen in Brca1-deficient cells.***

• Data generated as part of this grant and recently published in ***Cell***, (Callen, 2013), has shown that a 53BP1 phosphomutant, 53BP18A, comprising alanine substitutions of the eight most N-terminal S/TQ phosphorylation sites, mimics 53BP1 deficiency by restoring genome stability in BRCA1-deficient cells yet behaves like wild-type 53BP1 with respect to immunoglobulin class switch recombination (CSR).

53BP18A recruits RIF1 but fails to recruit the DDR protein PTIP to DSBs, and disruption of PTIP phenocopies 53BP18A. The work from this paper has allowed us to conclude that 53BP1 promotes productive CSR and suppresses mutagenic DNA repair through distinct phospho-dependent interactions with RIF1 and PTIP. The domain structure of 53BP1 and the different mutants used in this study are shown below along with a model that describes these findings (Fig. 4 below and Figs. 1 and 7 from Callen, ***Cell*** 2013)

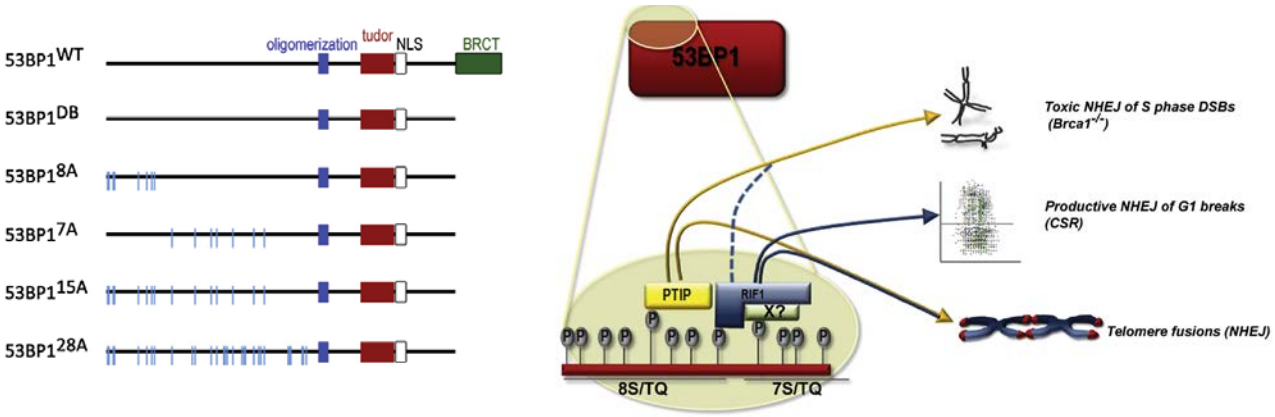

Fig. 4. Various 53BP1 constructs generated to describe the separation of function observed in 53BP1 (left panel). This separation of function ascribed to 53BP1 is illustrated in the model figure (right panel). Data supporting this model demonstrates that PTIP and RIF1 association with DSBs is dependent on distinct phosphorylation sites within 53BP1 (*Cell*, Callen, 2013).

Aim 3: ***Develop small molecule inhibitors of 53BP1 as possible lead compounds to inhibit Brca-mediated tumor formation. This highly ambitious project is ongoing.***

• We have recently obtained a GFP-53BP1 containing cell line (Stephen Kron, University of Chicago) that conditionally (dox inducible) expresses a minimal domain within 53BP1 (Fig. 5). This truncated form of 53BP1 forms robust foci following DNA damage and appears to recapitulate biological features observed with endogenous 53BP1 (Fig. 6).

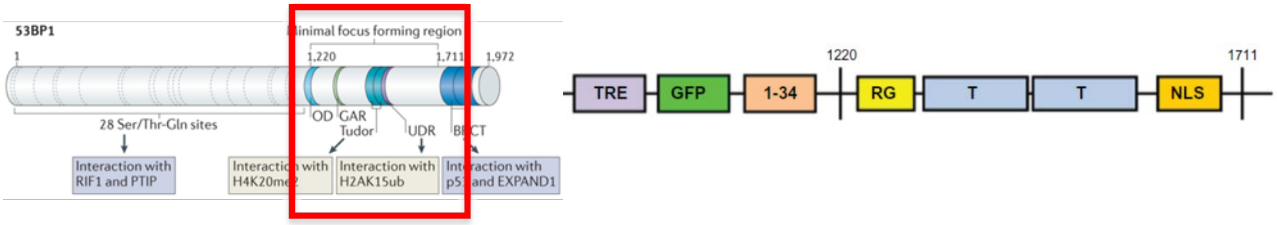

Fig. 5. Domain structure of the full-length 53BP1 protein. A minimal region (left panel, red square) has been fused with GFP and a generic nuclear localization signal. This truncated 53BP1 contains tandem Tudor domains (T) which have been demonstrated to interact with methylated histone residues on histone H4 (right panel).

• We have determined conditions for measuring 53BP1 foci using a GFP-based approach that elicits robust foci formation following treatment with the radiomimetic drug, neocarzinostatin (NCS). In collaboration with Dr. Marc Ferrer of the National Center for Advancing Translational Science and Jim McMahon of the Molecular targets Lab at NCI, we have been able to visualize foci using automated

high-throughput imaging platforms that will enable screening of the NCI Diversity Set of compounds. Lead compounds will be categorized as those that suppress the appearance of 53BP1 foci or enhance the chromatin association of 53BP1 following DNA damage. Currently we are optimizing this system with appropriate positive and negative controls (see Fig. 6 below). This work will enable us to conduct a screen of the NIH Diversity Set of small molecules, thereby enabling us to carry out a more detailed high-throughput approach detailed in Specific Aim 3.

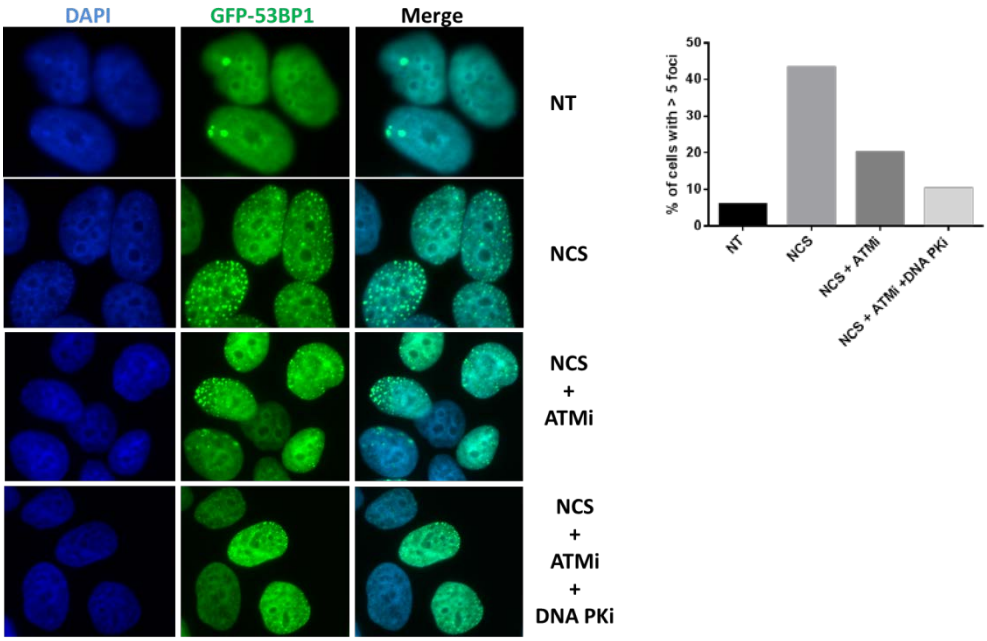

Fig. 6. A robust, cell-based foci-forming assay for screening 53BP1 inhibitors. GFP-53BP1 was induced with doxycycline for 48 hours followed by treatment (15 min) with the DNA double strand break inducing drug, neocarzinostatin (NCS). Short treatments with NCS induce the formation of numerous GFP-53BP1 foci (left panel, 2nd row). Foci formation is dramatically reduced by a 2hr pre-treatment with ATM inhibitors (ATMi) or the combined action of ATM and DNA PK inhibitors (left panel). A quantitation of the effect of inhibitors on foci formation is shown in the right panel.

Key Research Accomplishments

- Deletion of the DNA damage response gene RIF1 mimics 53BP1 deficiency with respect to increased resection and defective class switching.
- Deletion of the DNA damage response gene PTIP rescues homologous recombination in BRCA1-deficient cells.
- PTIP ablation confers PARPi and cis-platin resistance in BRCA1 and BRCA2-deficient cells.
- A systematic mutational analysis of phosphorylation sites within 53BP1 allows for functional segregation of domains within 53BP1 that interact with RIF1 and PTIP.
- Preliminary characterization of a DNA damage-inducible GFP-53BP1 foci forming cell line for high throughput screening of small molecule libraries.

Reportable Outcomes

Papers:
Two high-impact papers and two review papers have been published based on this work with the awardee, Dr. Andre Nussenzweig.

a. Di Virgilio, M., et al., ***Science*** 2013
b. Callen E., et al., ***Cell***, 2013
c. Daniel, J.A and Nussenzweig, A. ***Mol. Cell***, 2013
d. Bunting, S.F and Nussenzweig, A. ***Nat. Rev. Cancer***, 2013
e. One additional paper, based in part on conceptual and technical advances from this award, is in review in ***Nature***.

Invited talks:

1. March 3-6, 2014-Maintenance of Genome Stability Conference in St. Kitts- "Genome Stability during DNA Replication"
2. January 29-31, 2014- AACR Conference on Cancer Susceptibility and Cancer Susceptibility Syndromes in San Diego, CA-"Role of BRCA1 in genome stability"
3. December 10-14, 2013-CTRC-AACR- San Antonio Breast Cancer Symposium in Texas- "Mechanisms that maintain genome stability"
4. November 14, 2013-Fox Chase Cancer Center Distinguished Lecture Series –Philadelphia, PA- Title of talk, "Mechanisms that maintain genome stability."
5. September 16-17, 2013-University of Pennsylvania's Scientific Advisory Board and attending the 2013 Abramson Family Cancer Research Institute's Scientific Advisory Board Meeting-No Talk
6. June 12, 2013- 2012-2013 Samuel Lunenfeld Research Institute Seminar Series in Toronto, Canada-"Maintenance of Genome Stability."
7. May 29-30- Attend Scientific Advisory Board Meeting at the University of Copenhagen-No talk
8. May 27, 2013-Collaborative Research Center 655 from Cells to Tissues seminar series at the Max-Planck-Institute in Dresden, Germany-"Genome Stability during DNA Replication"
9. May 25, 2013- 5th Else Kroner-Fresenius Symposium on Adult Stem Cells in Aging, Diseases & Cancer in Eisenach, Germany-"Genome Stability during DNA Replication"
10. May 3, 2013- Chemical and Systems Biology Department Seminar Series at Stanford University- "Genome Stability during DNA Replication"
11. April 15, 2013- Spanish National Cancer Research Centre (CNIO) in Madrid, Spain- "Genome Instability during DNA Replication."
12. April 12, 2013-University of Zurich Cancer Mini-Symposium in Grindelwald, Switzerland- "Genome Stability during DNA Replication"
13. March 3-8, 2013- Keystone Symposia: Genetic Instability and DNA Repair in Fairmont Banff Springs, Alberta- "Identification of a novel class of early replicating fragile sites that contribute to genomic instability in B cell lymphomas"

Conclusions

Work generated in support of the DOD Idea grant has demonstrated that 53BP1 deletion promotes ATM-dependent processing of broken DNA ends to produce recombinogenic single-stranded DNA competent for HR. In addition to providing new mechanistic insight, this study has important therapeutic implications because loss of 53BP1 may be a mechanism by which BRCA1-mutant tumors develop

resistance to chemotherapy. Consistent with this, we found that loss of 53BP1 alleviates hypersensitivity of BRCA1- mutant cells to PARP inhibition. We have also begun to map the pathways that acts downstream of 53BP1 to inhibit HR: we discovered that the effector proteins RIF1 (M. Di Virgilio et al. ***Science*** 2013) and PTIP (Callen et al. ***Cell*** 2013) are recruited to sites of DNA damage via interactions with phosphorylated 53BP1, where they facilitate DNA repair in part by protecting DNA ends from resection.

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3. Daniel JA, Nussenzweig A. The AID-induced DNA damage response in chromatin. *Mol Cell*. 50(3):309-21, 2013. Review

4. Bunting SF, Nussenzweig A. End-joining, translocations and cancer. *Nat Rev Cancer*. 13(7):443-54, 2013. Review

Appendices

1. Di Virginio M, Callen E, Yamane A, Jankovic M, Gitlin AD, Feldhahn N, Resch W, Chait BT, Nussenzweig A, Casellas R, Robbiani DF, Nussenweig MC. Rif1 prevents resection of DNA breaks and promotes immunoglobulin class switching, *Science*, 339(6120): 711-5, 2013

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4. Bunting SF, Nussenzweig A. End-joining, translocations and cancer. *Nat Rev Cancer*. 13(7):443-54, 2013. Review

Rif1 Prevents Resection of DNA Breaks and Promotes Immunoglobulin Class Switching

Michela Di Virgilio *et al.*

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higher numbers from colon contents than was the nitrate respiration–deficient mutant (Fig. 3H and fig. S8B). Collectively, these data suggested that nitrate respiration conferred a marked growth advantage on commensal *E. coli* in the lumen of the inflamed gut.

The picture emerging from this study is that nitrate generated as a by-product of the host inflammatory response can be used by *E. coli*, and likely by other commensal Enterobacteriaceae, to edge out competing microbes that rely on fermentation to generate energy for growth. Obligate anaerobic microbes in the intestine compete for nutrients that are available for fermentation but cannot use nonfermentable nutrients (such as fermentation end products). The ability to degrade nonfermentable substrates probably enables *E. coli* to sidestep this competition, which explains the fitness advantage conferred by nitrate respiration in the inflamed gut. Through this mechanism, inflammation contributes to a bloom of nitrate-respiration–proficient Enterobacteriaceae, providing a plausible explanation for the dysbiosis associated with intestinal inflammation (*3*–*12*). This general principle might also influence the dynamics of host-associated bacterial communities outside the large bowel, as nitrate respiration confers a fitness advantage in the oxygen-poor and nitrate-rich environment of the cystic fibrosis airway (*21*).

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Supplementary Materials

www.sciencemag.org/cgi/content/full/339/6120/708/DC1
Materials and Methods
Figs. S1 to S11
Tables S1 and S2
References (*22*–*39*)

7 November 2012; accepted 5 December 2012
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Rif1 Prevents Resection of DNA Breaks and Promotes Immunoglobulin Class Switching

Michela Di Virgilio,[1] Elsa Callen,[3]* Arito Yamane,[4]* Wenzhu Zhang,[5]* Mila Jankovic,[1] Alexander D. Gitlin,[1] Niklas Feldhahn,[1] Wolfgang Resch,[4] Thiago Y. Oliveira,[1,6,7] Brian T. Chait,[5] André Nussenzweig,[3] Rafael Casellas,[4] Davide F. Robbiani,[1] Michel C. Nussenzweig[1,2]†

DNA double-strand breaks (DSBs) represent a threat to the genome because they can lead to the loss of genetic information and chromosome rearrangements. The DNA repair protein p53 binding protein 1 (53BP1) protects the genome by limiting nucleolytic processing of DSBs by a mechanism that requires its phosphorylation, but whether 53BP1 does so directly is not known. Here, we identify Rap1-interacting factor 1 (Rif1) as an ATM (ataxia-telangiectasia mutated) phosphorylation-dependent interactor of 53BP1 and show that absence of Rif1 results in 5′-3′ DNA-end resection in mice. Consistent with enhanced DNA resection, Rif1 deficiency impairs DNA repair in the G_1 and S phases of the cell cycle, interferes with class switch recombination in B lymphocytes, and leads to accumulation of chromosome DSBs.

The DNA damage response factor p53 binding protein 1 (53BP1) is a multidomain protein containing a chromatin-binding tudor domain, an oligomerization domain, tandem breast cancer 1 (BRCA1) C-terminal (BRCT) domains, and an N-terminal domain with 28 SQ/TQ potential phosphorylation sites for phosphatidylinositol 3-kinase–related kinases [PIKKs, ataxia-telangiectasia mutated (ATM)/ATM and Rad3-related/DNA-dependent protein kinase catalytic subunit (DNA-PKcs)] (*1*–*3*). 53BP1 contributes to DNA repair in several ways: This protein facilitates joining between intrachromosomal double-strand breaks (DSBs) at a distance (synapsis) (*4*–*7*), it enables heterochromatic DNA repair through relaxation of nucleosome compaction (*2*, *3*), and it protects DNA ends from resection and thereby favors repair of DSBs that occur in G_1 phase by nonhomologous end joining (NHEJ) (*4*, *5*, *8*). Consistent with its role in DNA-end protection, 53BP1 is essential for class switch recombination (CSR) in B lymphocytes (*9*, *10*).

Structure-function studies indicate that, besides the recruitment of 53BP1 to DNA ends, protection requires 53BP1 phosphorylation (*4*), but how this protective effect is mediated is unknown. To identify phosphorylation-dependent interactors of 53BP1, we applied stable isotope labeling by amino acids in cell culture (SILAC). *Trp53bp1*$^{-/-}$ (*Trp53bp1* encodes 53BP1) B cells were infected with retroviruses encoding a C-terminal deleted version of 53BP1 (53BP1^{DB}) or a phosphomutant in which all 28 N-terminal potential PIKK phosphorylation sites were mutated to alanine (53BP1^{DB28A}) (*4*), in media containing isotopically heavy (53BP1^{DB}) or light (53BP1^{DB28A}) lysine and arginine (fig. S1, A to C) (*11*).

Most proteins coprecipitating with 53BP1^{DB} and 53BP1^{DB28A} displayed a $H/(H+L)$ ratio of ~0.5 (H, heavy; L, light), which is characteristic of phospho-independent association (average of 0.57 ± 0.09, peptide count: at least four) (Fig. 1 and table S1). Many of these proteins are nonspecific contaminants, but others such as KRAB-associated protein 1 (KAP-1), dynein light chain LC8-type 1 (Dynll1), Nijmegen breakage syndrome 1 (Nbs1), and H2AX represent authentic phospho-independent 53BP1-interacting proteins (fig. S1D). Three proteins displayed an abundance ratio that was more than four standard deviations (SDs) above the mean, indicating that these proteins interact specifically

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with phosphorylated 53BP1: Pax interaction with transcription-activation domain protein-1 (Paxip1, or PTIP; 0.95), PTIP-associated protein 1 (Pa1; 0.97), and Rap1-interacting factor 1 (Rif1) (0.96) (Fig. 1 and figs. S1D and S2). PTIP was known to interact with 53BP1 in a phospho-dependent manner (*12*), whereas Pa1 and Rif1 were not.

Rif1 was originally identified in budding yeast as a protein with a key role in telomere length maintenance (*13*). However, in mammalian cells, Rif1 is not essential for telomere homeostasis, but has been assigned a number of different roles in maintaining genome stability, including participation in the DNA damage response (*14*–*16*), repair of S-phase DNA damage (*17*, *18*), and regulation of origin firing during DNA replication (*19*, *20*). However, the mechanism by which Rif1 might contribute to DNA repair and maintenance of genome stability is not known.

To confirm that Rif1 interaction with 53BP1 is dependent on phosphorylation, we performed Western blot analysis of Flag immunoprecipitates from lysates of irradiated *Trp53bp1*$^{-/-}$ B cells infected with retroviruses encoding 53BP1^{DB} or 53BP1^{DB28A}. Whereas Dynll1, a phospho-independent 53BP1 interactor (SILAC ratio: 0.55) (fig. S1D), coimmunoprecipitated with 53BP1^{DB} and 53BP1^{DB28A} to a similar extent (Fig. 2A), only 53BP1^{DB} coimmunoprecipitated with Rif1. We conclude that the interaction between 53BP1 and Rif1 is dependent on phosphorylation of 53BP1.

Ataxia-telangiectasia mutated phosphorylates 53BP1 in response to DSBs (*1*, *3*). To determine whether ATM induces DNA damage–dependent association between Rif1 and 53BP1, we compared irradiated and nonirradiated B cells in coimmunoprecipitation experiments. Although we detected small amounts of Rif1 in 53BP1^{DB} immunoprecipitates from unirradiated cells, this was increased by a factor of >3 after irradiation, and the increase was abrogated by treatment with the ATM inhibitor KU55933 (Fig. 2B). We conclude that Rif1 preferentially interacts with phosphorylated 53BP1 in a DNA damage- and ATM-dependent manner.

Rif1 is recruited to DNA damage foci by 53BP1 (*15*). To determine whether 53BP1 phosphorylation is required for Rif1 focus formation, we tested Rif1 foci in irradiated *Trp53bp1*$^{-/-}$ immortalized mouse embryonic fibroblasts (iMEFs), which were stably transduced with either 53BP1^{DB} or 53BP1^{DB28A}. Rif1 foci were readily detected and colocalized with 53BP1^{DB} (Fig. 2C). In contrast, although 53BP1^{DB28A} formed normal-appearing foci, Rif1 foci were rare and did not colocalize with 53BP1 (Fig. 2C). Furthermore, Rif1 recruitment to ionizing radiation–induced foci (IRIF) and colocalization with 53BP1 were abrogated in ATM-deficient but not DNA-PKcs–deficient iMEFs (fig. S3) (*15*). We conclude that Rif1 recruitment to DNA damage response foci is dependent on ATM-mediated 53BP1 phosphorylation.

The phosphorylation of 53BP1 is essential for CSR (*4*). To examine the role of Rif1 in joining DSBs during CSR, we conditionally ablated Rif1 in B cells using CD19^{Cre}, which is expressed specifically in B cells (*Rif1*$^{F/F}$*Cd19*$^{Cre/+}$ mice) (fig. S4, A to C). To induce CSR, B cells were activated with lipopolysaccharide (LPS) and interleukin-4 (IL-4) in vitro, and switching to immunoglobulin G1 (IgG1) or IgG3 was measured by flow cytometry. CSR to IgG1 and IgG3 was markedly reduced in *Rif1*$^{F/F}$*Cd19*$^{Cre/+}$ B cells, but less so than in *Trp53bp1*$^{-/-}$ controls (Fig. 3, A and B, and fig. S5). Switch junctions from *Rif1*$^{F/F}$*Cd19*$^{Cre/+}$ B cells were comparable to those from *Trp53bp1*$^{-/-}$ and wild-type controls (fig. S6) (*7*), which indicates that, similar to 53BP1 deficiency, absence of Rif1 does not alter the nature of productive CSR joining events.

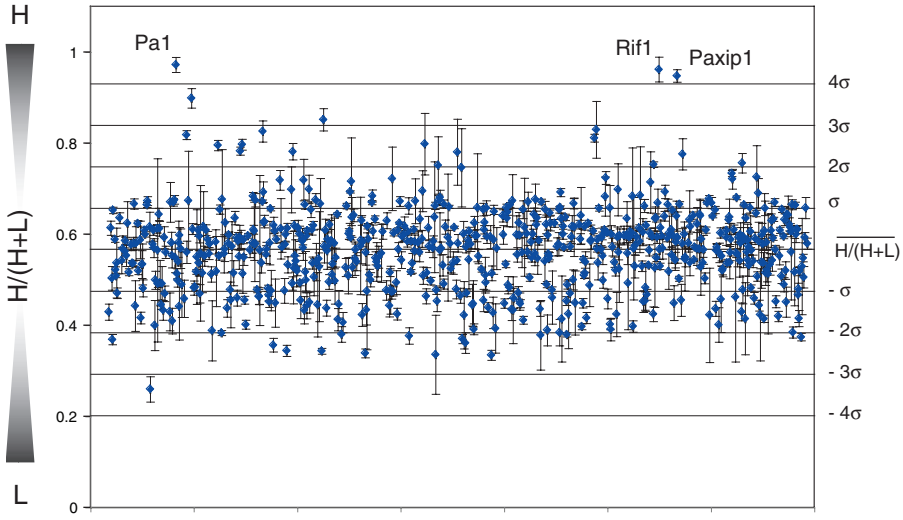
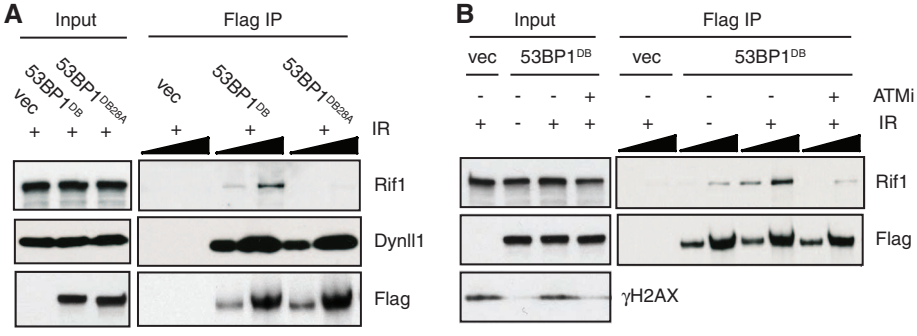


Fig. 1. Identification of phospho-dependent 53BP1 interactors. The graph shows the $H/(H + L)$ ratio distribution of proteins identified by SILAC. Error bars represent the SD of the $H/(H + L)$ mean value for all of the peptides identified for each individual protein (only proteins with at least four peptides were included). $\overline{H/(H + L)}$ and σ are the mean (0.57) and SD (0.09) of the distribution, respectively.



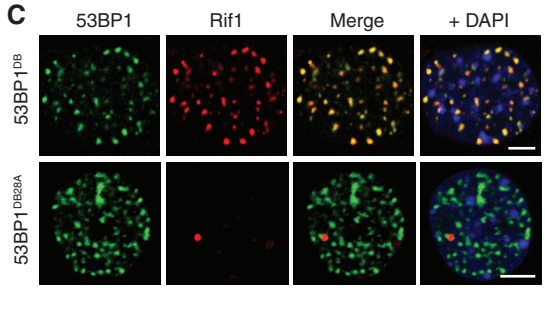


Fig. 2. Rif1 interaction with 53BP1 is dependent on phosphorylation, DNA damage, and ATM. (**A**) Western blot analysis of anti-Flag immunoprecipitates (IP) from irradiated (IR) *Trp53bp1*$^{-/-}$ B lymphocytes infected with empty vector (vec), 53BP1^{DB}, or 53BP1^{DB28A} virus. Triangles indicate threefold dilution. Data are representative of two independent experiments. (**B**) Western blot analysis of anti-Flag immunoprecipitates from *Trp53bp1*$^{-/-}$ B cells infected with empty vector or 53BP1^{DB}. Cells were either left untreated or irradiated [50 gray (Gy), 45-min recovery] in the presence or absence of the ATM kinase inhibitor KU55933 (ATMi). Triangles indicate threefold dilution. Data are representative of two independent experiments. (**C**) Immunofluorescent staining for 53BP1 (Flag) and Rif1 in irradiated *Trp53bp1*$^{-/-}$ iMEFs reconstituted with 53BP1^{DB} or 53BP1^{DB28A} retroviruses (*4*). Magnification, 100×; scale bars, 5 μm. Data are representative of two independent experiments. DAPI, 4′,6-diamidino-2-phenylindole.

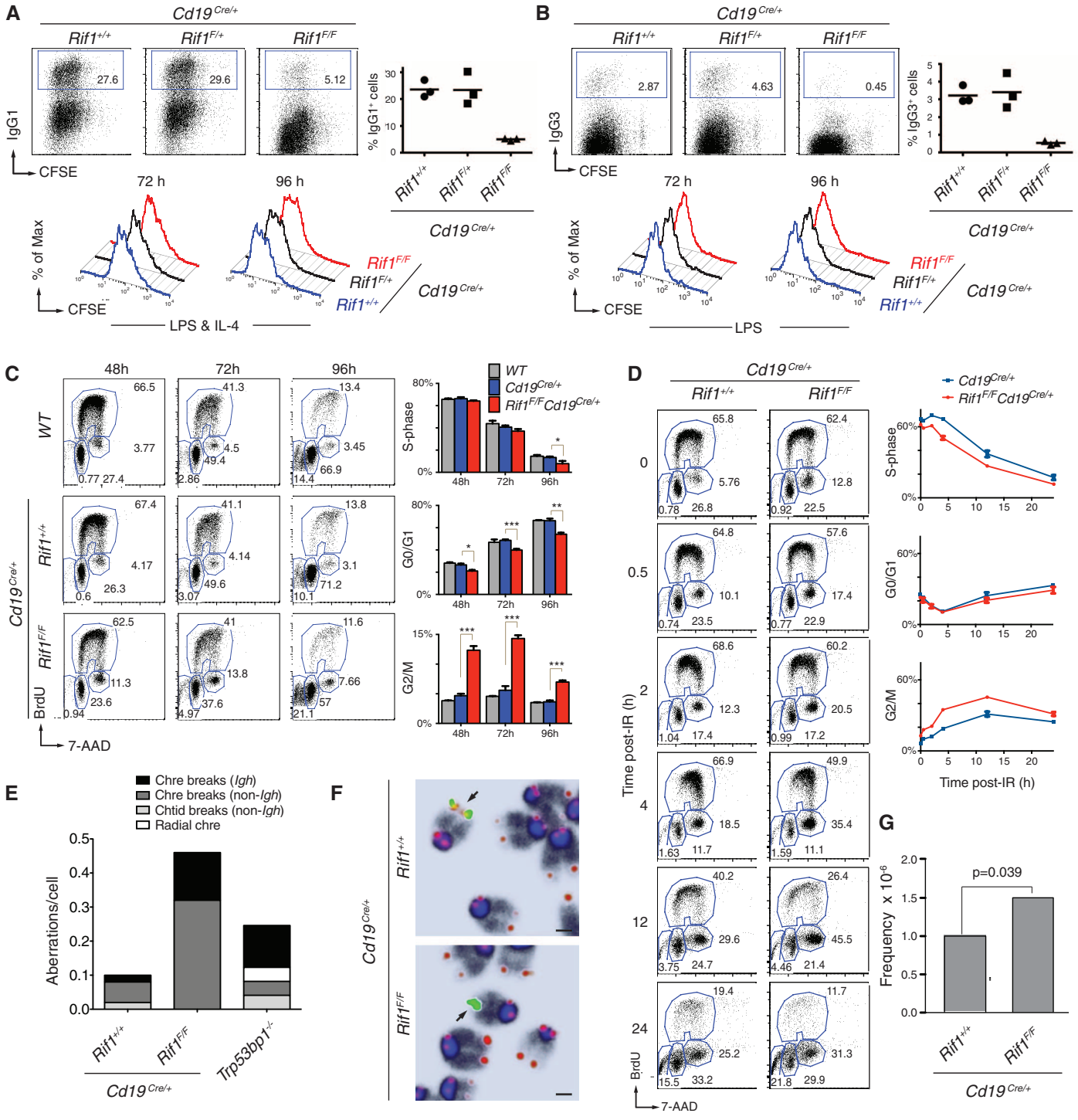


Fig. 3. Rif1 deficiency impairs CSR and causes *Igh* and genome instability in primary B cells. (**A**) (Left) CSR to IgG1 96 hours after stimulation of B lymphocytes with LPS and IL-4. (Right) Summary dot plot for three independent experiments (n = three mice per genotype). Mean values are: 23.6% for $Cd19^{Cre/+}$, 23.4% for $Rif1^{F/+}Cd19^{Cre/+}$, and 5.0% for $Rif1^{F/F}Cd19^{Cre/+}$ ($P < 0.008$ with the paired Student's t test). (Bottom) B cell proliferation by carboxyfluorescein succinimidyl ester (CFSE) dilution. Data are representative of three independent experiments. (**B**) Same as in (A) but for CSR to IgG3 after stimulation with LPS alone. Mean values are: 3.2% for $Cd19^{Cre/+}$, 3.4% for $Rif1^{F/+}Cd19^{Cre/+}$, and 0.5% for $Rif1^{F/F}Cd19^{Cre/+}$ ($P < 0.008$). (**C**) (Left) Cell cycle analysis of primary B cells after stimulation with LPS and IL-4. BrdU, 5-bromo-2′-deoxyuridine; 7-AAD, 7-amino-actinomycin D. (Right) Summary histograms for S, G_0/G_1, and G_2/M phase cells from two independent experiments (n = four mice per genotype). Error bars indicate SEM. * $0.01 < P < 0.05$, ** $0.001 < P < 0.01$, *** $P < 0.001$. WT, wild type. (**D**) (Left) Cell cycle analysis of LPS- and IL-4–stimulated splenocytes at the indicated times after irradiation (6 Gy). (Right) Summary graphs for S, G_0/G_1, and G_2/M phase cells from two independent experiments (n = three mice per genotype). Error bars indicate SD. (**E**) Analysis of genomic instability in metaphases from B cell cultures. Chtid, chromatid; Chre, chromosome. Data are representative of two independent experiments (n = 50 metaphases analyzed per genotype per experiment). (**F**) Examples of *Igh*-associated aberrations in $Rif1^{F/F}Cd19^{Cre/+}$ B cells. Chromosomes were hybridized with an *Igh* Cα probe (green; centromeric of Cγ1) and a telomere sequence-specific probe (red) and were counterstained with DAPI (dark blue/black). Arrows indicate *Igh* Cα/telomeric signal on chromosome 12. Magnification, 63×; scale bars, 1 μm. (**G**) Frequency of *c-myc/Igh* translocations in activated B cells. The graph shows combined results from three mice per genotype.

A similar CSR defect was also obtained by conditionally deleting Rif1 with 4-hydroxytamoxifen (4HT) in *Rif1*$^{F/F}$*ROSA26*$^{Cre\text{-}ERT2/+}$ B cells (fig. S7). Finally, short hairpin RNA–mediated partial down-regulation of CtBP-interacting protein (CtIP), which interacts with Rif1 (fig. S8C) and has been implicated in processing of DNA ends (*21*, *22*), resulted in a very small but reproducible increase in CSR (fig. S8, A and B). Thus, Rif1 is essential for normal CSR, and CtIP may not be the only factor that contributes to end processing in Rif1-deficient B cells.

Class switch recombination requires cell division, activation-induced cytidine deaminase (AID) expression, and *Igh* germline transcription (*23*). There are conflicting reports that Rif1 is required for proliferation in MEFs, but not in DT40 B cells (*17*, *18*). We found that cell division profiles of *Rif1*$^{F/F}$*Cd19*$^{Cre/+}$ and 4HT-treated *Rif1*$^{F/F}$*ROSA26*$^{Cre\text{-}ERT2/+}$ B cells were indistinguishable from controls (Fig. 3, A and B; and fig. S7, A, C, E, and G), indicating that Rif1 is dispensable for B cell proliferation in vitro. Finally, AID mRNA and protein expression and *Igh* germline transcription were not affected by Rif1 deletion (fig. S4, B and D).

We next examined the role of Rif1 in cell cycle progression in primary B cells. We found no major differences in the percentage of cells in G_0/G_1 and S phases (Fig. 3C). However, the number of cells in G_2/M phase was increased approximately twofold in the absence of Rif1 (2.64-, 2.56-, and 1.91-fold at 48, 72, and 96 hours, respectively) (Fig. 3C). We obtained similar results with the use of *Rif1*$^{F/F}$*ROSA26*$^{Cre\text{-}ERT2/+}$ B cells treated with 4HT (fig. S7, H and I).

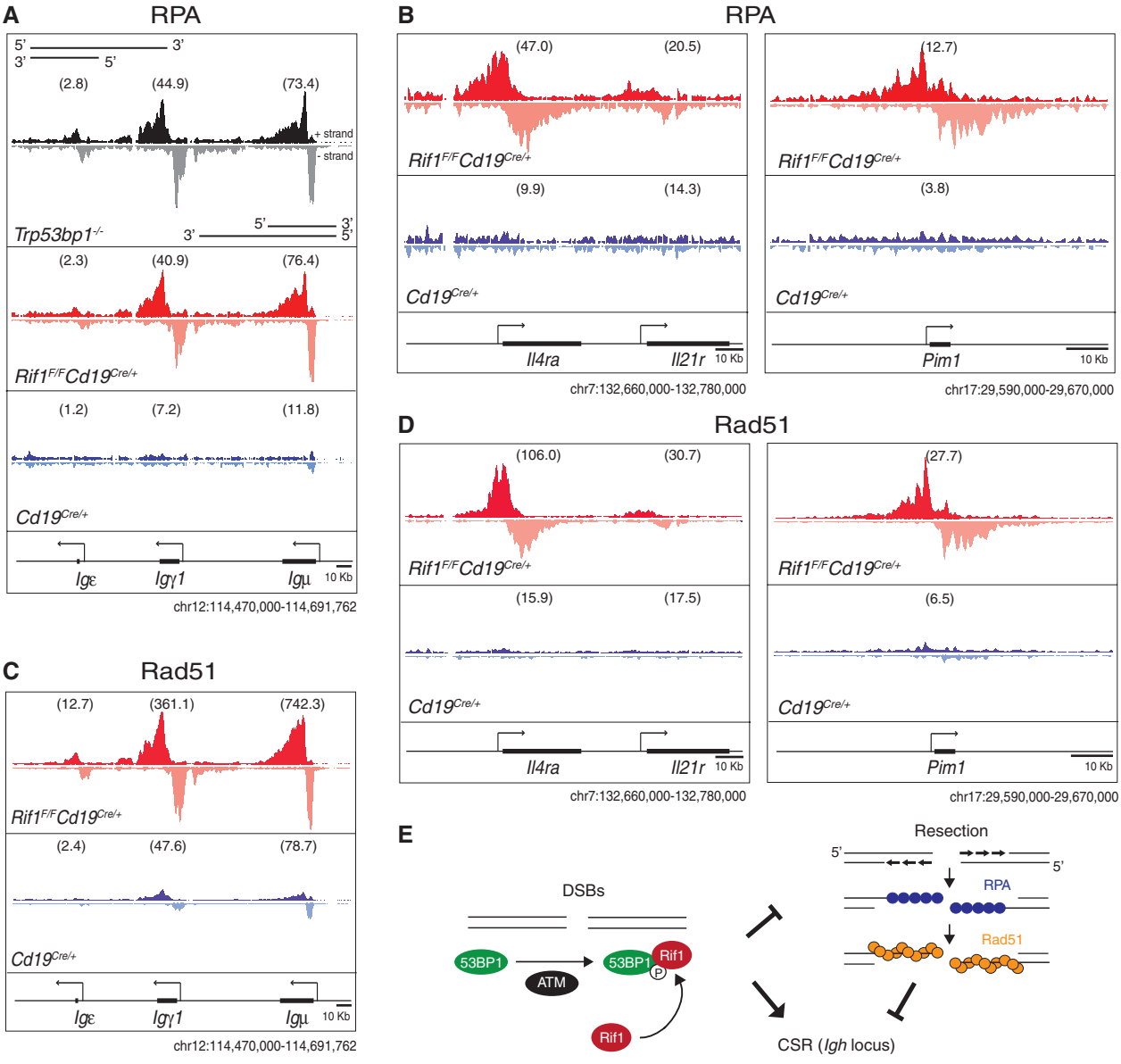


Fig. 4. Rif1 prevents resection of DNA ends at sites of AID-induced DNA damage. (**A** to **D**) RPA and Rad51 occupancy at the *Igh* locus (A and C) and at non-*Igh* AID targets genes (B and D) in B cells activated to undergo class switching. ChIP-seq libraries were resolved into upper (+) and lower (-) DNA strands to show RPA and Rad51 association with sense and antisense strands. Within a specified genomic window, graphs have the same scale and show tag density. Deep-sequencing samples were normalized per library size, and tags per million values were calculated for each genic region, as indicated in the supplementary materials and methods and shown in parenthesis. Data are representative of two independent experiments for RPA ChIP-seq and one for Rad51. (**E**) Model of Rif1 recruitment and DNA-end protection at DSBs. DNA damage activates ATM, which phosphorylates many targets, including 53BP1. This event recruits Rif1 to 53BP1 at the DSB, where it inhibits DNA resection. The extensive resection in the absence of Rif1 impairs CSR at the *Igh* locus. P, phosphate.

Furthermore, irradiation increases the accumulation of $Rif1^{F/F}Cd19^{Cre/+}$ B cells in G2/M phase (Fig. 3D). In addition, $Trp53bp1^{-/-}$ iMEFs expressing $53BP1^{DB28A}$, which did not recruit Rif1 to IRIF (Fig. 2C), exhibited delayed progression through S phase following DNA damage with accumulation of cells in G_2 phase after irradiation (fig. S9).

Accumulation of cells in G_2/M phase may reflect the persistence of unrepaired DNA damage in a fraction of Rif1-deficient cells. To investigate this possibility, we analyzed metaphase spreads from B cells dividing in response to LPS and IL-4 in vitro. These cells express AID, which produces DSBs in *Igh* and, less frequently at off-target sites throughout the genome, in the G_1 phase of the cell cycle (*24*–*26*). Chromosomal aberrations were increased in $Rif1^{F/F}Cd19^{Cre/+}$ B cells compared to controls (Fig. 3E), with many localized to the *Igh* locus (Fig. 3E). Consistent with the observation that *Igh* is targeted by AID in the G_1 phase of the cell cycle, all of the *Igh* breaks were chromosome breaks (Fig. 3, E and F). Interestingly, the frequency of *c-myc/Igh* translocations is moderately increased in $Rif1^{F/F}Cd19^{Cre/+}$ B cells; however, the breakpoint distribution was similar to the $Cd19^{Cre/+}$ control (1.5×10^{-6} versus 1.0×10^{-6} in the control; $P = 0.039$) (Fig. 3G and fig. S10). We conclude that in the absence of Rif1, DSBs fail to be resolved efficiently in the G_1, S, or G_2 phases, which leads to increased levels of genomic instability, including chromosome breaks at *Igh* and translocations in dividing B cells.

In the absence of 53BP1, DSBs produced by AID at the *Igh* locus accumulate the single-stranded DNA-binding replication protein A complex (RPA) as a result of increased DNA-end resection (*24*). To determine if Rif1 is required for DNA-end protection by 53BP1, we performed RPA–chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq) experiments on $Rif1^{F/F}Cd19^{Cre/+}$ and control B cells. Ablation of Rif1 was indistinguishable from the loss of 53BP1 in that in its absence, RPA decorates the *Igh* locus asymmetrically, in a manner consistent with 5′-3′ resection (Fig. 4A) (*27*). In addition, absence of Rif1 also results in RPA accumulation at non-*Igh* genes, such as *Il4ra* and *Pim1*, that are damaged by AID in G_1 phase (Fig. 4B) (*24*, *25*). Rad51 is the recombinase that mediates repair of DSBs by homologous recombination in S/G_2/M phase (*22*). To confirm that Rif1 prevents resection that takes place in S phase, we monitored Rad51 accumulation in activated B cells by ChIP-seq. Loss of Rif1 was indistinguishable from the loss of 53BP1 (*27*), in that it led to asymmetric Rad51 accumulation at sites of AID-inflicted DNA damage (Fig. 4, C and D). We conclude that in the absence of Rif1, AID-induced DSBs incurred in G_1 phase persist and undergo extensive 5′-3′ DNA-end resection in S/G_2/M phase, as measured by RPA and Rad51 accumulation.

A role for Rif1 in maintenance of genome stability and protection of DNA ends against resection is consistent with its phosphorylation-dependent recruitment to the N-terminal domain of 53BP1 (*4*). 53BP1 facilitates DNA repair and prevents DNA-end resection during CSR. In the absence of 53BP1, AID-induced DSBs are resolved inefficiently in G_1 phase, leading to chromosome breaks, *Igh* instability, and resolution by alternative NHEJ or homologous recombination instead of classical NHEJ (*4*, *8*, *27*). Our experiments show that in the absence of Rif1, 53BP1 is insufficient to promote genomic stability or mediate efficient *Igh* repair, DNA-end protection, or CSR. Thus, these 53BP1 activities require Rif1 recruitment to the phosphorylated N terminus of 53BP1. Rif1 is likely to have additional functions beyond 53BP1, CSR, and DNA-end protection because although $Trp53bp1^{-/-}$ mice are viable, Rif1 deletion is lethal (*17*). Indeed, Rif1 is believed to play a role in the repair of S-phase DNA damage (*17*, *18*), as well as in the regulation of replication timing (*19*, *20*, *28*). Analogously, additional CSR factor(s) may exist downstream of 53BP1, as class switching in Rif1-deficienct B cells is significantly higher than in $Trp53bp1^{-/-}$.

In summary, our data are consistent with a model in which ATM-mediated phosphorylation of 53BP1 recruits Rif1 to sites of DNA damage, where it facilitates DNA repair in part by protecting DNA ends from resection (Fig. 4E). In the absence of Rif1, DNA breaks incurred in G_1 phase fail to be repaired by NHEJ and undergo extensive 5′-3′ end resection, resulting in the accumulation of chromosome breaks and genome instability.

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Supplementary Materials

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Materials and Methods
Figs. S1 to S10
Table S1
References (*29*–*49*)

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Cell

53BP1 Mediates Productive and Mutagenic DNA Repair through Distinct Phosphoprotein Interactions

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SUMMARY

The DNA damage response (DDR) protein 53BP1 protects DNA ends from excessive resection in G1, and thereby favors repair by nonhomologous end-joining (NHEJ) as opposed to homologous recombination (HR). During S phase, BRCA1 antagonizes 53BP1 to promote HR. The pro-NHEJ and antirecombinase functions of 53BP1 are mediated in part by RIF1, the only known factor that requires 53BP1 phosphorylation for its recruitment to double-strand breaks (DSBs). Here, we show that a 53BP1 phosphomutant, 53BP1^{8A}, comprising alanine substitutions of the eight most N-terminal S/TQ phosphorylation sites, mimics 53BP1 deficiency by restoring genome stability in BRCA1-deficient cells yet behaves like wild-type 53BP1 with respect to immunoglobulin class switch recombination (CSR). 53BP1^{8A} recruits RIF1 but fails to recruit the DDR protein PTIP to DSBs, and disruption of PTIP phenocopies 53BP1^{8A}. We conclude that 53BP1 promotes productive CSR and suppresses mutagenic DNA repair through distinct phosphodependent interactions with RIF1 and PTIP.

INTRODUCTION

Class switch recombination (CSR) is initiated by activation-induced cytidine deaminase (AID), which generates multiple double-strand breaks (DSBs) at highly repetitive immunoglobulin (Ig) switch regions. Paired distal DSBs are then rejoined by nonhomologous end-joining (NHEJ), thereby replacing Igμ by a downstream constant region (Igγ, Igε, or Igα). Alternatively, if DSBs persist, a homology-driven pathway that involves resection of repetitive switch regions, can repair DSBs locally. Such abortive "intraswitch" recombination events are increased at the expense of CSR in the absence of 53BP1(Reina-San-Martin et al., 2007), a key suppressor of end resection (Bothmer et al., 2010; Bouwman et al., 2010; Bunting et al., 2010; Cao et al., 2009; Difilippantonio et al., 2008).

In addition to its productive effect on CSR, 53BP1 blocks DNA ends from resection in BRCA1-deficient cells, leading to toxic radial chromosomes that arise from NHEJ (Bouwman et al., 2010; Bunting et al., 2010, 2012; Cao et al., 2009). Deletion of 53BP1 leads to deposition of homologous recombination (HR) factors RPA and RAD51 on single-strand DNA, which, in the case of recombining switch regions, promotes intraswitch recombination (Yamane et al., 2013) and, in the setting of BRCA1 deficiency, restores HR (Bouwman et al., 2010; Bunting et al., 2010; Cao et al., 2009). Thus, DNA end protection by 53BP1 is critical for CSR in G1 but can unleash genome instability in S phase.

In addition to DNA end-blocking activities that disfavor HR and thereby promote NHEJ, 53BP1 has been suggested to directly mediate long-range chromosomal interactions and DSB mobility that facilitates the juxtaposition of distal DNA ends. These activities are believed to be responsible for 53BP1's ability to support recombination of DSB ends that are far apart during V(D)J recombination and class switch recombination (Callén et al., 2007b; Difilippantonio et al., 2008) and to fuse uncapped telomeric DNA ends (Dimitrova et al., 2008). Both pro-NHEJ and

anti-HR functions require the direct physical association of 53BP1 with DNA ends but also necessitate the DSB-induced phosphorylation of its N-terminal ATM/ATR kinase sites (Bothmer et al., 2011; Ward et al., 2006).

The DNA damage response (DDR) protein RIF1 was recently identified as an essential factor recruited by phosphorylated 53BP1 to promote NHEJ and block HR (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Feng et al., 2013; Zimmermann et al., 2013). Like 53BP1, RIF1 is required for CSR (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013). Although the NHEJ of dysfunctional telomeres is abrogated in cells lacking 53BP1 or in cells expressing $53BP1^{28A}$(Lottersberger et al., 2013), an allele harboring alanine substitutions at all 28 N-terminal ATM/ATR kinase phosphorylation targets sites, loss of RIF1 has considerably milder defect (Zimmermann et al., 2013). Moreover, although the generation of toxic radial chromosomes in BRCA1-deficient cells is prevented in *53BP1*$^{-/-}$ or in $53BP1^{28A}$ mutant cells (Bothmer et al., 2011; Bouwman et al., 2010; Bunting et al., 2012; Bunting et al., 2010), the loss of RIF1 only partially rescues HR in BRCA1-deficient cells (Escribano-Díaz et al., 2013; Feng et al., 2013; Zimmermann et al., 2013). This suggests that additional phosphorylation-dependent but RIF1-independent activities of 53BP1 might regulate the balance between HR and NHEJ.

PTIP is a ubiquitously expressed nuclear protein that associates constitutively with two of the known histone methyltransferases that catalyze trimethylation of histone H3 at lysine 4 (H3K4me3), MLL3, and MLL4 (Cho et al., 2007; Patel et al., 2007). In addition to its well-established role in transcription initiation, a separate pool of PTIP functions in an unknown capacity in the DDR (Gong et al., 2009). Indeed, PTIP has been implicated in both HR (Wang et al., 2010) and NHEJ (Callen et al., 2012). PTIP is recruited to DSBs by its tandem BRCT (BRCA1 carboxyl-terminal) domains (Manke et al., 2003; Yu et al., 2003), which associate with the serine 25 phosphorylation site within the N terminus of 53BP1 (Munoz et al., 2007). In contrast to RIF1, PTIP recruitment to DSBs was reported to be 53BP1 and ATM independent (Gong et al., 2009; Jowsey et al., 2004; Munoz et al., 2007). Thus, the mechanism by which PTIP is recruited to DSBs, its role in DSB repair, and the physiological significance of PTIP interaction with 53BP1 remain unclear. Here, we show that PTIP is required for 53BP1-mediated inhibition of HR in BRCA1-deficient cells but is dispensable for 53BP1-initiated DSB repair during productive CSR. Thus, RIF1 and PTIP separate 53BP1 functions in productive and pathologic DSB repair.

RESULTS

A Separation of Function Mutation in 53BP1

To determine whether 53BP1's activities in NHEJ and HR are distinct, we compared $53BP1^{8A}$, which disrupts phosphorylation of the eight N-terminal ATM/ATR target sites (Figure 1A), to the $53BP1^{DB}$ allele, which is indistinguishable from WT 53BP1 in all functional aspects (Bothmer et al., 2011). To assay for CSR, BRCA1/53BP1-deficient B cells were transduced with wild-type and 53BP1 mutant proteins by retroviral infection after activation with lipopolysaccharide (LPS) and interleukin-4 (IL4). As expected, $53BP1^{DB}$ fully complemented the CSR defects (Figure 1B) and produced high levels of genome instability in PARPi-treated BRCA1/53BP1-deficient cells (Figure 1C) (Bothmer et al., 2011). Surprisingly, despite rescuing CSR, the $53BP1^{8A}$ allele failed to promote genome instability in PARPi-treated BRCA1/53BP1-deficient cells above the levels observed in controls (Figure 1C). This effect was not due to differences in the expression levels of 53BP1 (Figure 1D) or in the recruitment of 53BP1 and RIF1 to DSBs (Figure 1E). Similar to B cells, BRCA1/53BP1-deficient MEFs complemented with $53BP1^{DB}$ were hypersensitive to PARPi, whereas $53BP1^{8A}$ transduced MEFs were not (Figure S1 available online). Thus, the mechanism by which 53BP1 promotes CSR and blocks HR in BRCA1-deficient cells is distinct. Moreover, the recruitment of RIF1 is insufficient to induce genome instability in PARPi-treated BRCA1-deficient cells.

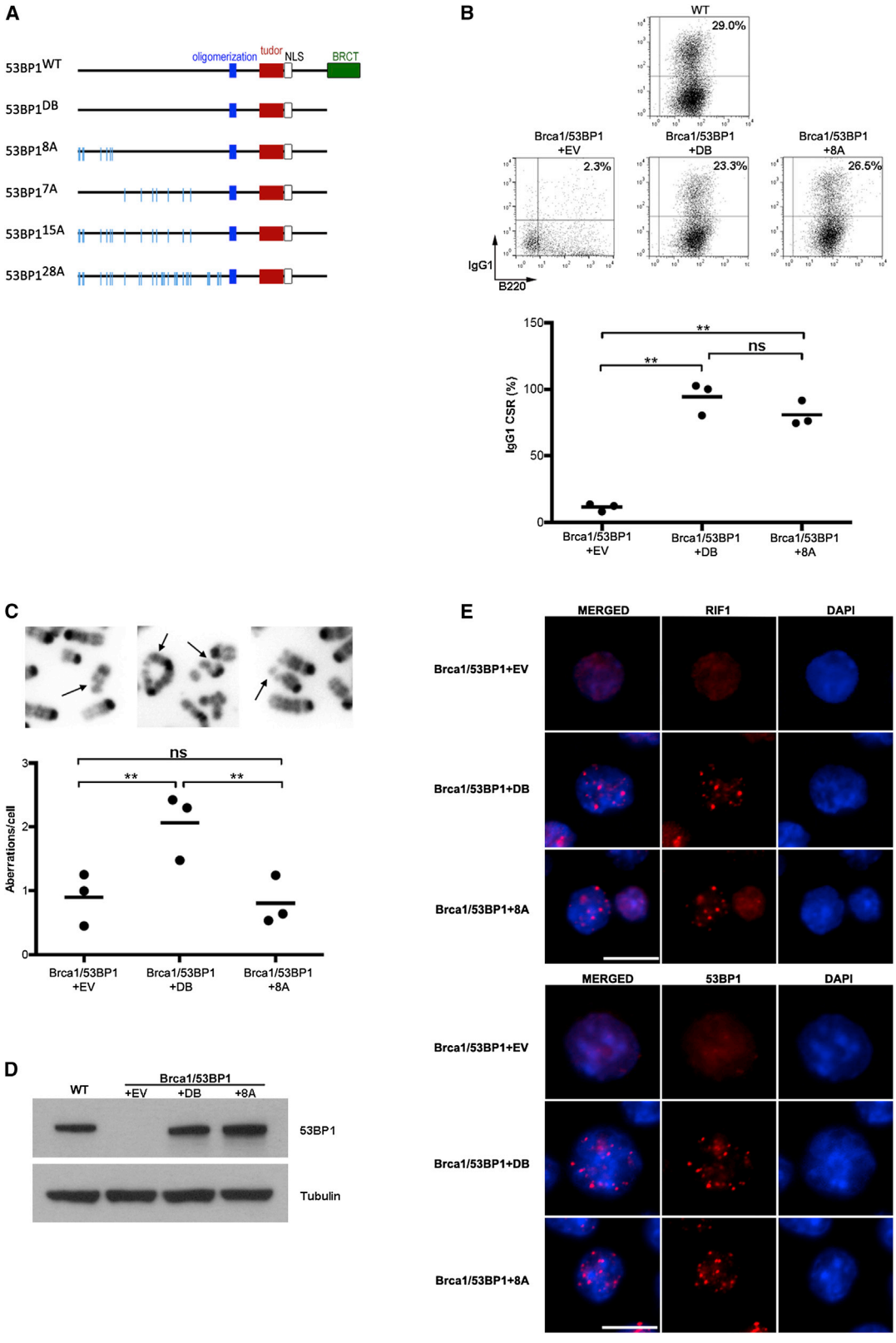
Role of PTIP in the DNA Damage Response

Upon DNA damage, PTIP binds to the serine 25 residue within the N terminus of 53BP1(Munoz et al., 2007), which is located within the eight N-terminal sites mutated in $53BP1^{8A}$. Consistent with this, immunoprecipitation analysis revealed that PTIP association with 53BP1 after irradiation was abrogated in cells expressing S25A-harboring mutants $53BP1^{8A}$, $53BP1^{15A}$, or $53BP1^{28A}$ (Figures 2A and S2A). In contrast, the damage-induced 53BP1/PTIP interaction was maintained in the $53BP1^{7A}$ mutant, comprising alanine substitutions of 7 S/TQ phosphorylation sites C terminus of those mutated in $53BP1^{8A}$ (Figures 1A and S2A).

To explore the function of PTIP in the DDR, we asked whether PTIP-deficient cells are sensitive to DNA damaging agents that are predominantly repaired by HR (Sonoda et al., 2006). WT and *PTIP*$^{-/-}$ MEFs were exposed to either cisplatin, camptothecin, or PARPi, all of which sensitize HR-deficient cells (Bryant et al., 2005; Farmer et al., 2005). Each of these agents induced a similar level of chromosomal aberrations and reduction in cell survival in WT and *PTIP*$^{-/-}$ MEFs (Figures 2B, 2C, and S2B). In contrast, *PTIP*$^{-/-}$ MEFs were sensitive to irradiation (IR) (Figures 2B, 2C, and S2B) (Gong et al., 2009; Jowsey et al., 2004; Munoz et al., 2007). Moreover, $53BP1^{8A}$ MEFs exhibited increased genome instability and reduced cell survival following IR (Figures S2C and S2D). To examine the recruitment of HR proteins to DSBs, we evaluated BRCA1, RAD51, and γ-H2AX foci formation after IR in WT and *PTIP*$^{-/-}$ MEFs. All of these factors were normally recruited to DSBs in PTIP-deficient cells (Figure S2E). Moreover, 53BP1 also formed robust foci in the absence of PTIP (Figure S2E). Thus, *PTIP*$^{-/-}$ MEFs are tolerant to agents that are highly toxic to HR-deficient cells and the recruitment of several factors implicated in DSB repair is intact in the absence of PTIP. Nevertheless, both *PTIP*$^{-/-}$ and $53BP1^{8A}$ MEFs are sensitive to IR.

PTIP Is Dispensable for NHEJ during CSR but Is Required for NHEJ of Dysfunctional Telomeres

To explore the role of PTIP in NHEJ, we first assayed CSR. Deletion of PTIP in B cells leads to a defect in class switching to IgG3, IgG2b, and IgG1 (Daniel et al., 2010; Schwab et al., 2011). By recruiting an MLL-like methyltransferase complex to the switch regions of these isotypes, PTIP promotes histone modifications



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and transcription initiation of IgG3/IgG2b/IgG1 germline switch regions, which are necessary for AID targeting (Daniel et al., 2010; Schwab et al., 2011). However, PTIP does not affect transcription at Igμ and Igε (Daniel et al., 2010), indicating that PTIP-associated methyltransferase complex promotes the accessibility of some but not all switch loci. To distinguish between PTIP's effects on transcription versus DSB repair, we compared CSR to IgG1 and IgE on day 5 after stimulation with αCD40+IL4 as described (Wesemann et al., 2011). As expected *PTIP*$^{f/f}$ *CD19*^{CRE} (*PTIP*$^{-/-}$) B cells displayed a defect in switching to IgG1 (Figures 3A and 3B), which is consistent with decreased *Igγ1* germline transcription (Daniel et al., 2010; Schwab et al., 2011). However, there was no defect in IgE germline transcription (Daniel et al., 2010) or IgE CSR in PTIP-deficient cells (Figures 3A and 3B). Indeed, IgE CSR was consistently higher in the absence of PTIP, likely because Sγ1 is no longer a target for AID. In contrast to *PTIP*$^{-/-}$, ablation of RIF1 in *Rif1*$^{f/f}$ CD19^{CRE} (*RIF1*$^{-/-}$) B cells impaired CSR to both IgG1 and IgE (Figures 3A and 3B). We conclude that loss of PTIP phenocopies the 53BP1^{8A} mutant allele in that neither has a significant impact on NHEJ during CSR.

An alternative end-joining pathway can catalyze substantial CSR end-joining to IgG1 and IgE even in the absence of classical NHEJ (Boboila et al., 2010). Loss of PTIP leads to IR sensitivity but tolerance to agents that are repaired by HR. We therefore speculated that PTIP might function in other reactions besides CSR that might rely on classical NHEJ, such as the fusion of dysfunctional telomeres. When the shelterin factor TRF2 is removed, deprotected telomeres trigger ATM-dependent phosphorylation of 53BP1, and the ends are processed by NHEJ to generate chromosome fusions (Celli et al., 2006; Rai et al., 2010; Zimmermann et al., 2013). Because ATM-dependent phosphorylation of 53BP1 is also required for interaction between 53BP1 and PTIP (Figures 2A and S2A) (Jowsey et al., 2004; Manke et al., 2003), we asked whether PTIP promotes NHEJ-mediated fusion of deprotected telomeres. To address this, we uncapped telomeres in SV40-immortalized WT and *PTIP*$^{-/-}$ MEFs by removing TRF2 with short hairpin RNA against TRF2 (Rai et al., 2010). Upon TRF2 depletion we observed a similar level of phosphorylation of the ATM target KAP-1 in WT and *PTIP*$^{-/-}$ MEFs, as measured by quantitative flow cytometry (Figure 4A). Consistent with this, there was an accumulation of cytologically discernable telomere-induced DNA damage foci (TIFs) containing γ-H2AX in WT and *PTIP*$^{-/-}$ cells (Figure 4B). Despite a robust DNA damage response and activation of ATM, the frequency of end-end chromosomal fusions was reduced by 2.8-fold in *PTIP*$^{-/-}$ MEFs relative to WT (Figures 4C and 4D). Whereas 42% of WT cells bearing fusions had more than 30% of their chromosome ends fused, only 13% of PTIP KO cells had greater than 30% of their ends fused (Figure 4E). Thus, PTIP deficiency results in a reduction in the number of long-chain telomere fusions when telomeres are deprotected. We conclude that PTIP contributes to the NHEJ of dysfunctional telomeres.

PTIP Promotes Genome Instability in BRCA1-Deficient Cells

In contrast to 53BP1, loss of RIF1 only partially reverses the chromosomal aberrations and hypersensitivity produced by PARPi treatment of BRCA1-deficient cells (Escribano-Díaz et al., 2013; Zimmermann et al., 2013). To determine whether PTIP could overcome the HR defects in BRCA1-deficient cells, we crossed *PTIP*$^{f/f}$ and *BRCA1*$^{f(Δ11)/f(Δ11)}$ mice with CD19 CRE transgenic mice to simultaneously delete PTIP and exon 11 of BRCA1 in primary B lymphocytes. When unchallenged, *BRCA1*$^{+/+}$*PTIP*$^{+/+}$ CD19^{CRE} (WT), *BRCA1*$^{f(Δ11)/f(Δ11)}$ CD19^{CRE} (*BRCA1*$^{-/-}$), *PTIP*$^{f/f}$ CD19^{CRE} (*PTIP*$^{-/-}$), and *BRCA1*$^{f(Δ11)/f(Δ11)}$*PTIP*$^{f/f}$ CD19^{CRE} (*BRCA1*$^{-/-}$*PTIP*$^{-/-}$) doubly deficient B cells divided normally as determined by carboxyfluorescein succinimidyl ester (CFSE) dye dilution (Figure 5A) and cell-cycle distribution (Figure 5B). Treatment with PARPi did not impair the proliferation of WT or *PTIP*$^{-/-}$ B cells (see also Figures 2B and 2C); however, *BRCA1*$^{-/-}$ cells underwent fewer divisions over the course of 72 hr (Figure 5A). In contrast, loss of PTIP completely reversed the *BRCA1*$^{-/-}$ growth defect (Figure 5A). Strikingly, although PARPi treatment generated chromatid breaks, chromosome breaks, and radial chromosomes in BRCA1-deficient cells (Bunting et al., 2010), *BRCA1*$^{-/-}$*PTIP*$^{-/-}$ B cells were insensitive to PARPi (Figure 5C). Thus, ablation of PTIP phenocopies both the 53BP1^{8A} mutation (Figure 1C) and 53BP1 deficiency (Bouwman et al., 2010; Bunting et al., 2010; Cao et al., 2009) in that it promotes genome stability and survival in BRCA1 mutant cells.

Loss of PTIP Increases HR in BRCA1 Mutant Cells by Promoting DSB Resection

BRCA1 and RAD51 function in a common HR pathway that promotes RAD51-mediated DNA strand exchange (Bhattacharyya et al., 2000; Moynahan et al., 1999; Scully et al., 1997). Loss of 53BP1 rescues RAD51 foci formation and HR in

Figure 1. Characterization of a Separation of Function Mutant 53BP1

(A) Diagram of the 53BP1 retroviral constructs used. Hash marks indicate location of substituted S/TQ sites.

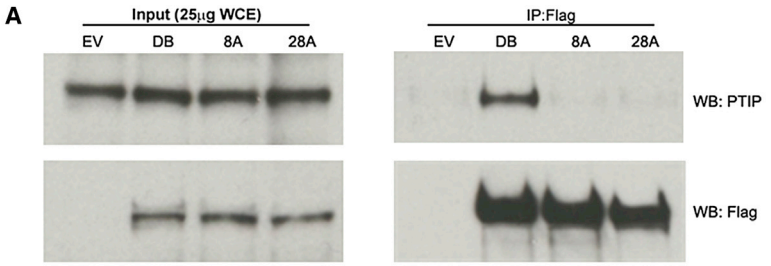
(B) Top: Representative flow cytometry plots measuring CSR after stimulation of WT and *BRCA1*$^{-/-}$*53BP1*$^{-/-}$ B cells infected with retroviruses expressing 53BP1^{DB} (amino acids 1–1710), the N-terminal mutant 53BP1^{8A} or empty vector (EV). Numbers represent the percentages of IgG1 switched cells. B220 is a B cell marker. Bottom: Dot plot indicating IgG1 CSR as a percentage of WT value in the same experiment. Three independent experiments are shown. **$p < 0.001$ (two-tailed unpaired t test); BRCA1/53BP1+DB versus BRCA1/53BP1+8A, $p > 0.1$, which is not significant (ns).

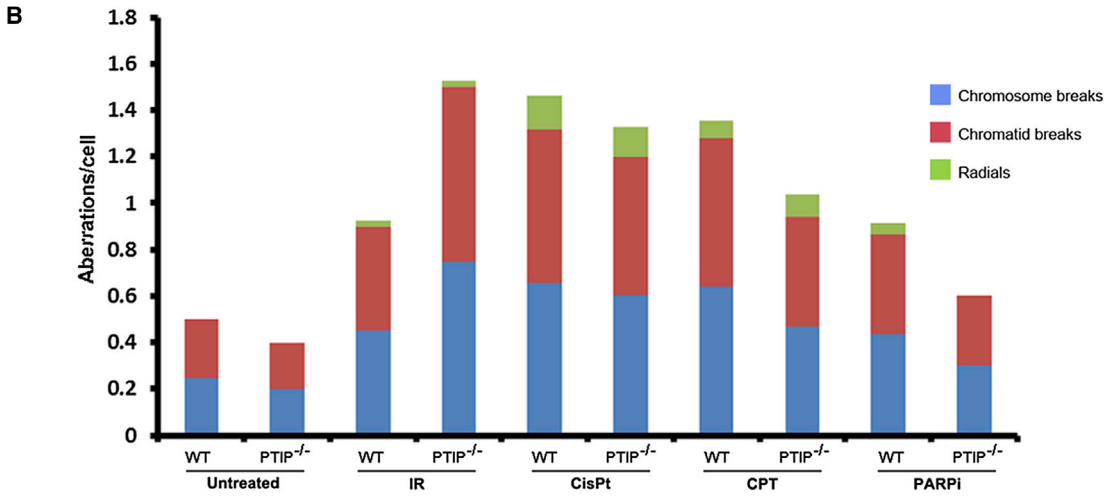
(C) *BRCA1*$^{-/-}$*53BP1*$^{-/-}$ B cells were reconstituted with empty vector, 53BP1^{DB} and 53BP1^{8A} retroviruses and treated with PARPi. The arrows indicate representative images of aberrant chromosomes. Dot plot indicates the total aberrations per cell in three independent experiments. At least 50 metaphases were analyzed for each genotype in each experiment. **$p < 0.01$ (two-tailed unpaired t test); ns: not significant.

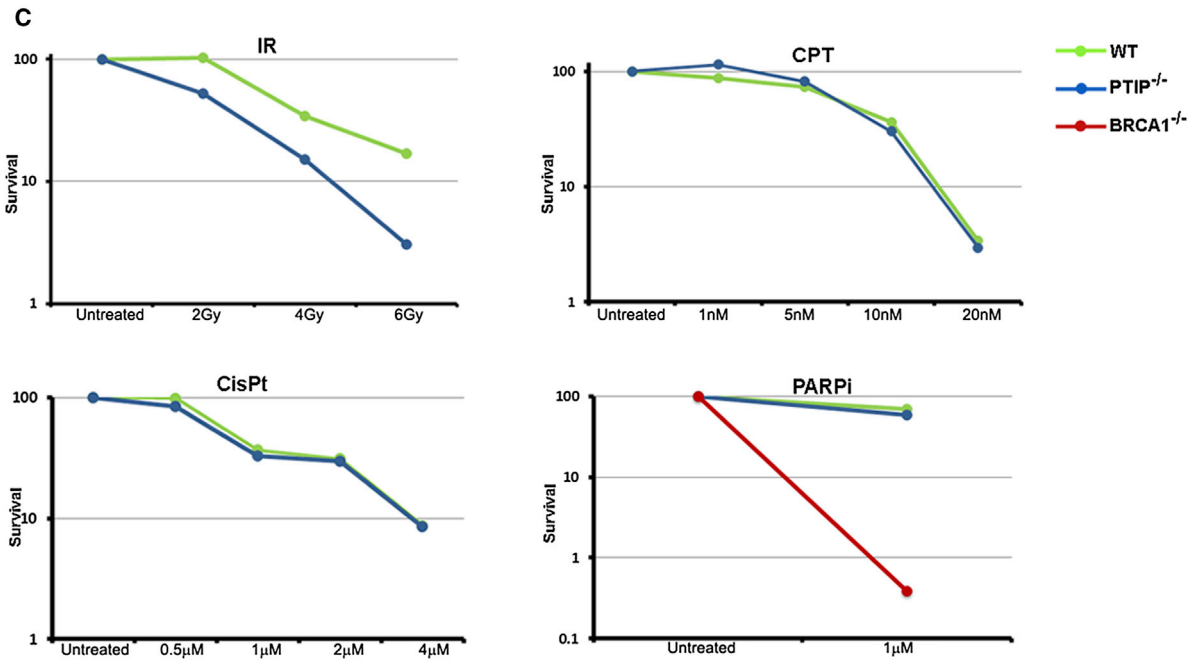
(D) Western blot analysis of 53BP1 expression in WT B cells and *BRCA1*$^{-/-}$*53BP1*$^{-/-}$ B cells stimulated and infected with empty vector, 53BP1^{DB}, or 53BP1^{8A}.

(E) *BRCA1*$^{-/-}$*53BP1*$^{-/-}$ B cells infected with EV, 53BP1^{DB} or 53BP1^{8A} retroviruses were assayed for IRIF (10 Gy, 2 hr recovery) for RIF1 (red, top), and 53BP1 (red, bottom). Cells were counterstained with DAPI (blue). Scale bar, 10 μm.

See also Figure S1.







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BRCA1-deficient cells (Bouwman et al., 2010; Bunting et al., 2010). To explore whether PTIP deficiency also promotes HR in BRCA1-deficient cells, we irradiated WT, *BRCA1*$^{-/-}$, *PTIP*$^{-/-}$, and *BRCA1*$^{-/-}$*PTIP*$^{-/-}$B cells and measured the frequency of immunofluorescent RAD51 foci. All mutant cells proliferated similarly to WT over the course of 3 days (Figure 5A), and as expected, RAD51 foci were reduced in IR-treated *BRCA1*$^{-/-}$ cells (Figure 5D). However, in *PTIP*$^{-/-}$ cells, the frequency of RAD51 foci was greater than WT, and RAD51 foci were normalized to WT levels in *BRCA1*$^{-/-}$*PTIP*$^{-/-}$ B cells (Figure 5D). These results suggest that loss of PTIP reverses the HR defect in BRCA1-deficient cells, thereby explaining the insensitivity of *BRCA1*$^{-/-}$ *PTIP*$^{-/-}$ B cells to PARPi.

Loss of PTIP might promote RAD51 foci formation by allowing increased resection of DSBs; this is similar to what happens with the loss of 53BP1 (Bunting et al., 2010; Difilippantonio et al., 2008). Because 5′→3′ DSB end resection produces RPA-coated single-strand DNA, we monitored RPA foci formation by high content microscopy. Irradiated *PTIP*$^{-/-}$ cells exhibited a significant increase in the mean number of RPA foci per cell relative to WT (Figure 5E); moreover, the fraction of *PTIP*$^{-/-}$ cells that had more than 15 RPA foci following IR was approximately 2-fold greater than WT (Figure 5E). Thus, PTIP limits the amount of chromatin bound RPA at IR-induced DSBs.

PTIP Recruitment to DSBs Promotes Radial Chromosomes in BRCA1-Deficient Cells

PTIP is a subunit of the MLL3/4 methyltransferase complex and promotes histone H3 lysine 4 trimethylation and transcription initiation at specific promoters, such as the Sγ3/Sγ1 switch regions of the *Igh* locus (Daniel et al., 2010) (Figure S3A). To determine whether transcription of DDR genes is altered by PTIP ablation, we profiled the transcriptome of WT and *PTIP*$^{-/-}$ B cells. Overall, there were 471 RefSeq annotated genes that were deregulated by more than 5-fold in *PTIP*$^{-/-}$ versus WT (Figure S3B). However, HR and NHEJ DNA damage response genes were not among deregulated pathways (Figures S3B and S3C). This suggests that the functions of PTIP in suppressing HR might be unrelated to its role in transcriptional regulation.

To determine whether PTIP recruitment to DSBs is essential for its effects on HR, we made use of a point mutation in the BRCT domain 3 (W663R) of PTIP that selectively blocks its interaction with 53BP1 (Gong et al., 2009; Munoz et al., 2007) and is unable to form foci (Figure S4A) (Daniel et al., 2010) but retains PTIP association with the MLL3/4 complex, which is dependent on BRCT (domains 5 and 6) (Patel et al., 2007). *BRCA1*$^{-/-}$ *PTIP*$^{-/-}$ B cells were infected with PTIP^{WT} and PTIP^{W663R} encoding retroviruses, treated with PARPi, and monitored for chromosomal damage (Figures 5F and S4B). Whereas PTIP^{WT} expression in *BRCA1*$^{-/-}$*PTIP*$^{-/-}$ cells led to an increase in the number of chromosomal radials relative to uninfected cells, *BRCA1*$^{-/-}$*PTIP*$^{-/-}$ cells transduced with PTIP^{W663R} remained insensitive (Figures 5F and S4B). Thus, PTIP recruitment to DSBs is necessary to block HR in BRCA1-deficient cells.

Recruitment of PTIP to DSBs Is Dependent on the Eight Most N-Terminal S/TQ Phosphorylation Sites of 53BP1

To explore the mechanism of PTIP recruitment to DSBs, we expressed FLAG-tagged PTIP in WT, *53BP1*$^{-/-}$, and *ATM*$^{-/-}$ MEFs and irradiated them with 10 Gy (Figure 6A). Although PTIP ionizing-irradiation-induced foci (IRIF) were detectable in nearly all WT cells, PTIP IRIF formation was impaired in the absence of 53BP1 or ATM (Figure 6A). Measurements of colocalization coefficients of γ-H2AX (a marker of the DNA breaks) with PTIP in irradiated WT, *53BP1*$^{-/-}$, and *ATM*$^{-/-}$ MEFs revealed that 80% of γ-H2AX foci in WT cells contained PTIP, whereas less than 15% and 10% of γ-H2AX foci in the *53BP1*$^{-/-}$ and *ATM*$^{-/-}$ cells, respectively, contained PTIP. Consistent with these findings, PTIP IRIF was highly sensitive to pharmacological inhibition of ATM (ATMi), less sensitive to ATRi treatment, and insensitive to DNA-PKi. (Figure S5). These findings contrast with previous reports suggesting that PTIP, 53BP1, and ATM are independently recruited to DSBs (Gong et al., 2009; Jowsey et al., 2004; Munoz et al., 2007). Because available PTIP antibodies are unable to detect endogenous PTIP foci, we used laser microirradiation to generate DSBs in WT, *53BP1*$^{-/-}$, and *ATM*$^{-/-}$ MEFs. In WT cells, PTIP was recruited to laser scissors-induced DSBs, which colocalized with γ-H2AX (Figure 6B). Consistent with our analysis of IRIF, PTIP recruitment to DNA damage sites was 53BP1 and ATM dependent (Figure 6B). Moreover, PTIP failed to be recruited to DSBs in *53BP1*$^{-/-}$ MEFs reconstituted with a mutant protein lacking all 28 N-terminal S/TQ phosphorylation sites of 53BP1, 53BP1^{28A} (Figure 6B). We conclude that ATM-dependent phosphorylation of 53BP1 is necessary for PTIP recruitment to DSBs.

Given that RIF1 is also recruited to DSBs in a 53BP1- and ATM-dependent manner (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Feng et al., 2013; Silverman et al., 2004; Zimmermann et al., 2013), we next monitored the codependency of PTIP and RIF1 for localization to DNA damage foci (Figure 6C). We found that 82% of PTIP IRIF colocalized

Figure 2. Response of PTIP to Different DNA Damaging Agents

(A) *53BP1*$^{-/-}$ B cells were reconstituted with empty vector, 53BP1^{DB}, 53BP1^{8A}, or 53BP1^{28A} retroviruses that were FLAG-tagged. Cells were irradiated (10 Gy, 45 min recovery) and immunoprecipitation was performed with anti-FLAG antibodies. Western blot analysis of PTIP and FLAG are shown for input (left) and immunoprecipitated protein (right).

(B) Isogenic immortalized WT and *PTIP*$^{-/-}$ MEFs were either untreated or treated with irradiation (IR, 2 Gy), cisplatin (CisPt, 0.5 μM), camptothecin (CPT, 10 nM) or PARP inhibitor (PARPi, 1 μM) and chromosomal aberrations (chromatid breaks, chromosome breaks, and radials) were quantified in at least 50 metaphase spreads for each genotype and each treatment. Data from an independent experiment is shown in Figure S2B.

(C) WT (green lines) and *PTIP*$^{-/-}$ (blue line) MEFs were treated with different doses of the above drugs, and colony formation was quantified relative to colonies formed in untreated cells from the same genotype. An experiment performed in parallel demonstrated that 1 μM PARPi treatment is toxic for BRCA1 mutant MEFs (red line).

See also Figure S2.

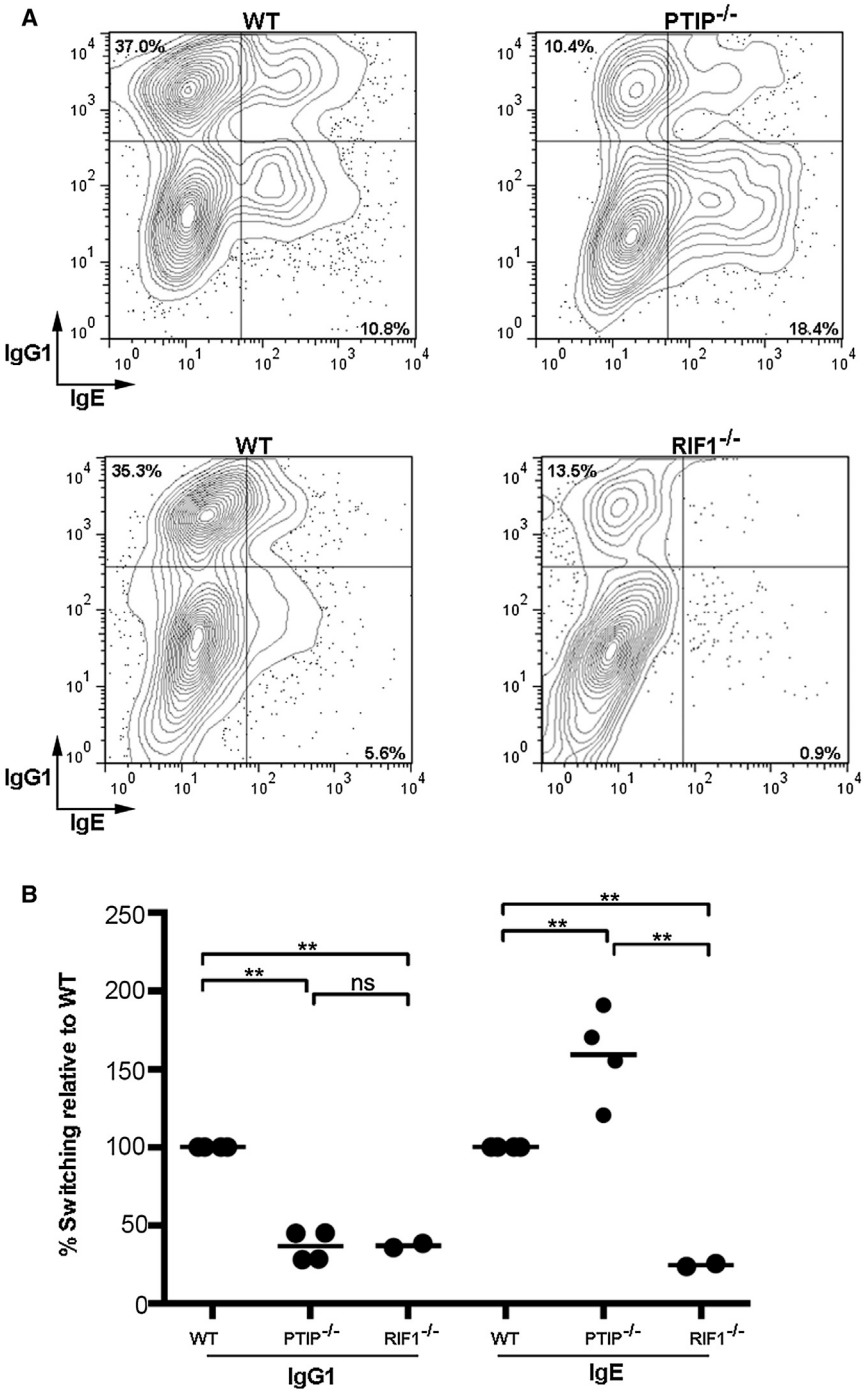

Figure 3. PTIP Is Dispensable for CSR to IgE

PTIP$^{f/f}$*CD19*^{CRE} (*PTIP*$^{-/-}$), (*Rif1*$^{f/f}$*CD19*^{CRE}) *RIF1*$^{-/-}$ and littermate WT B cells were stimulated with αCD40 plus IL-4 and analyzed for IgG1 and IgE CSR on day 5.

(A) Representative flow cytometry plots. The percentages of IgG1 switched cells (upper-left quadrant) and IgE switched cells (lower-right quadrant) is indicated.

(B) Dot plot indicates IgG1 and IgE CSR in *PTIP*$^{-/-}$ and *RIF1*$^{-/-}$ as a percentage of the WT value in the same experiment. **p < 0.01 (two-tailed unpaired t test); ns: not significant.

with RIF1 foci and 78% of RIF1 colocalized with PTIP foci (Figure 6C, n > 800 foci). However, RIF1 was recruited to DNA damage sites in *PTIP*$^{-/-}$ MEFs (Figure 6D) and vice versa (Figure 6E). Thus, RIF1 and PTIP are independently recruited to IRIF in a phospho-53BP1-dependent manner.

To further define the residues required for recruitment to phospho-53BP1, we examined PTIP and RIF1 recruitment in 53BP1^{DB}, 53BP1^{8A}, and 53BP1^{7A} mutant MEFs (Figures 2A and S2A). Whereas expression of 53BP1^{DB} in *53BP1*$^{-/-}$ MEFs reconstituted PTIP IRIF (Figures 7A and S6A), PTIP recruitment to DSBs was abrogated in 53BP1^{8A} MEFs (Figures 7A and S6A). By contrast, RIF1 recruitment was independent of these eight most N terminus phosphorylation sites on 53BP1, partially dependent on the seven S/TQ phosphorylation sites C terminus to 53BP1^{8A}, and abrogated in 53BP1^{15A} mutant cells that lack all 8S/TQ and 7S/TQ phosphorylation sites (Figures 7A–7C and S6B). Thus, PTIP and RIF1 exhibit distinct phosphorylation-dependent interactions with 53BP1 that guide them to DSBs. The association of PTIP with the 8S/TQ sites on 53BP1 upon DNA damage (Figures 2A, 7, and S6A) likely explains why loss of PTIP phenocopies 53BP1^{8A} with respect to CSR, irradiation sensitivity, and reversal of genome instability in BRCA1-deficient cells.

DISCUSSION

Regulation of DSB Repair Choice

53BP1 and BRCA1 play a critical role in channeling DSBs into either NHEJ or HR. 53BP1 promotes NHEJ in G1 by tethering DSBs together and by protecting these ends from exonuclease processing (Bothmer et al., 2010; Difilippantonio et al., 2008). In S phase, the inhibitory effect of 53BP1 on resection is antagonized by BRCA1 (Bouwman et al., 2010; Bunting et al., 2010). Loss of BRCA1 results in a shift toward a mutagenic NHEJ pathway that results in chromosomal abnormalities, tumorigenesis, and embryonic lethality, but all of these phenotypes are relieved by 53BP1 deletion (Bouwman et al., 2010; Bunting et al., 2010; Cao et al., 2009). In contrast, loss of classical NHEJ proteins (e.g., Ku, Ligase IV, DNA-PKcs) does not overcome the HR defects associated with BRCA1 deficiency (Bunting et al., 2012; Bunting et al., 2010), perhaps because these factors play a more limited role

in repressing 5′-3′ resection (Bunting et al., 2012; Sfeir and de Lange, 2012). Despite the striking rescue of BRCA1 deficiency, disrupting 53BP1 does not reverse the DNA repair defects associated with downstream mediators of the HR reaction (e.g., XRCC2, BRCA2, or PALB2) (Bouwman et al., 2010; Bowman-Colin et al., 2013; Bunting et al., 2010). Thus, 53BP1 and BRCA1 oppose each other during critical initial stages of DSB repair before commitment to repair the ends by NHEJ or HR.

Mechanism of PTIP and RIF1 Association with 53BP1

The molecular events that are required for 53BP1 to promote the ligation of DNA ends during CSR and the aberrant chromosomal rearrangements in BRCA1 mutant cells were previously thought to be identical. Surprisingly our data suggest that the pro-NHEJ and anti-HR functions of 53BP1 are in fact distinct and separable activities that nevertheless require 53BP1 phosphorylation. These complementary aspects of 53BP1's activities are mediated by the independent recruitment of RIF1 and PTIP, respectively, to phosphorylated 53BP1.

PTIP contains BRCT domains that interact directly with phosphorylated 53BP1 (Manke et al., 2003; Munoz et al., 2007). In contrast, RIF1 does not contain a known phosphorecognition motif, and it remains unclear how ATM-dependent phosphorylation facilitates RIF1 association with 53BP1. RIF1 may associate with 53BP1 directly or through interactions with effector molecules that contain BRCT phosphobinding modules (Figure 7D). Based on the observation that there is no detectable defect in RIF1 foci in 53BP1^{8A} cells (Figures 7A and S6B), we suspected that a major RIF1-interaction motif would reside C terminus of the 8S/TQ PTIP interaction sites. Consistent with this, the 53BP1^{7A} C-terminal mutant exhibits a reduction in RIF1 IRIF (Figures 7A–7C) and CSR (Bothmer et al., 2011). RIF1 IRIF and CSR are further reduced in 53BP1^{15A} mutant cells that lack 8S/TQ and 7S/TQ sites (Figures 7A and 7B) (Bothmer et al., 2011), suggesting that both regions contribute to RIF1 interactions with 53BP1 (Figure 7D). If so, we would predict some degree of competition between PTIP and RIF1 binding to 53BP1. Consistent with this, we have found an increased association between PTIP and 53BP1 in response to DNA damage in RIF1-deficient cells (Figure S6C). Thus, distinct from PTIP, RIF1 association with 53BP1 occurs via multidomain interactions (Figure 7D).

Role of PTIP and RIF1 in DSB Resection

Deletion of either PTIP or RIF1 leads to increased resection (Figure 5E) (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Feng et al., 2013; Zimmermann et al., 2013). However, whereas PTIP ablation rescues HR in BRCA1-deficient cells and is largely dispensable for NHEJ during CSR, RIF1 is essential for CSR and only partially contributes to the HR defects in BRCA1-deficient cells (Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Feng et al., 2013; Zimmermann et al., 2013). How can these observations be reconciled? One possibility is that distinct S/TQ kinase target sites in 53BP1 are phosphorylated during CSR in G1 and during replication fork collapse in S, resulting in independent recruitment of the two factors to DNA ends in distinct phases of the cell cycle. Consistent with this idea, it was reported that the localization of RIF1 to DSBs is mainly restricted to G1 and is suppressed by BRCA1 in S/G2 (Chapman et al., 2013; Escribano-Díaz et al., 2013; Feng et al., 2013). However, our finding that PTIP and RIF1 colocalize in the majority of irradiated cells and that both proteins form IRIF during G1 and S/G2 (Figure S7) indicates that PTIP and RIF1 are not recruited to DSBs in distinct cell-cycle phases.

Another possibility is that PTIP and RIF1 sites on 53BP1 are equally phosphorylated during the cell cycle but that these proteins might make the DSB-proximal chromatin refractory to a distinct set of nucleases. For example, initial DNA end resection is mediated by MRE11/RAD50/NBS1 and CTIP, whereas DNA2, EXO1, and BLM carry out more extensive resection (Symington and Gautier, 2011), and RIF1 appears to be involved in protection against initial but not sustained resection (Feng et al., 2013). In this model, the level of resection supported by loss of RIF1 would be insufficient for complete rescue of HR in *BRCA1*-deficient cells, which might require more extensive 3′ single-strand tails. In contrast, ablation of PTIP supports the sustained resection required for the rescue of HR in *BRCA1*-deficient cells. Thus, RIF1 and PTIP may block different steps in resection or distinct nucleases that mediate HR.

Role of PTIP and RIF1 in Telomeric End-Joining

Depending on the nature of the break, RIF1 and PTIP might cooperate to promote NHEJ. For example, PTIP and RIF1 deficiency both result in IR sensitivity (Figures 2B and 2C) (Feng et al., 2013), and defective NHEJ of dysfunctional telomeres (Figure 4) (Chapman et al., 2013; Zimmermann et al., 2013). It has been demonstrated that 53BP1 has RIF1-independent roles in promoting telomeric end-joining, evidenced by the considerably higher frequency of telomeric fusions in *RIF1*$^{-/-}$*TRF2*$^{-/-}$ versus *53BP1*$^{-/-}$*TRF2*$^{-/-}$ or *53BP1*^{28A}*TRF2*$^{-/-}$ MEFs (Lottersberger et al., 2013; Zimmermann et al., 2013). This RIF1-independent but phospho-53BP1-dependent function at telomeres has been linked to the induction of chromosome mobility (Zimmermann et al., 2013), which increases the probability that DNA ends fuse. Because PTIP binds to DSBs in a 53BP1-dependent but RIF1-independent manner, it is possible that this 53BP1-dependent/RIF1-independent increase in telomere mobility is mediated by PTIP.

Implications for Cancer Therapy

The identification of separation of function mutations that selectively disrupt antirecombination functions of 53BP1 during replication fork collapse and CSR may open up new therapeutic opportunities. Breast cancers arising in BRCA1 mutation carriers frequently show low levels of 53BP1 expression (Bouwman et al., 2010), which might result in resistance to PARPi therapy, a promising strategy for treating HR-deficient tumors (Bryant et al., 2005; Farmer et al., 2005). Consistent with this, 53BP1 was lost in a fraction of BRCA1-deficient mouse mammary tumors that acquired PARPi resistance in vivo (Jaspers et al., 2013). Interestingly, a fraction of PARPi-resistant tumors restored HR yet did not lose 53BP1. We speculate that PTIP mutation might emerge as a novel causal factor in PARPi resistance of BRCA1-deficient mammary tumors that restore HR. With respect to intervention, our study also suggests that it might be possible to increase HR in BRCA1 heterozygous carriers

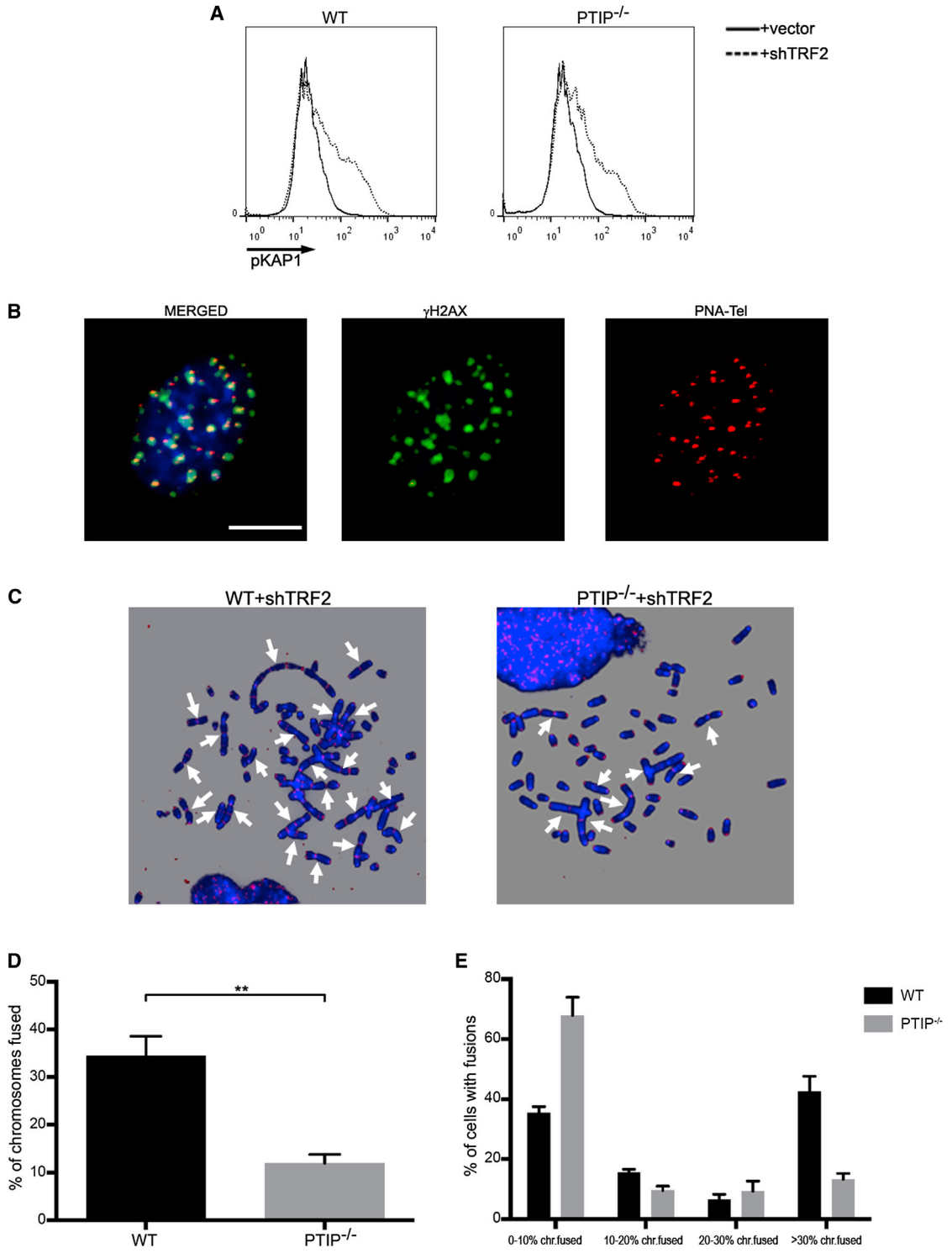

Figure 4. PTIP Is Required for NHEJ of Dysfunctional Telomeres

(A) WT and *PTIP*$^{-/-}$ MEFs were infected with a retrovirus expressing either an empty vector or shRNA against TRF2 (shTRF2), and phosphorylated KAP1 (pKAP1) levels were measured by flow cytometry.

(B) γ-H2AX (green) in telomere-dysfunction-induced foci (TIF) generated in shTRF2-infected WT cells. PNA probe is shown in red, and images are merged on top of DAPI (blue). Scale bar, 10 μm.

(C) Representative images of a metaphase spread from WT and *PTIP*$^{-/-}$ MEFs infected with shTRF2. Telomere fusions are visualized by a telomeric PNA probe (red) and DAPI (blue). Arrows point to representative telomeric fusions.

(legend continued on next page)

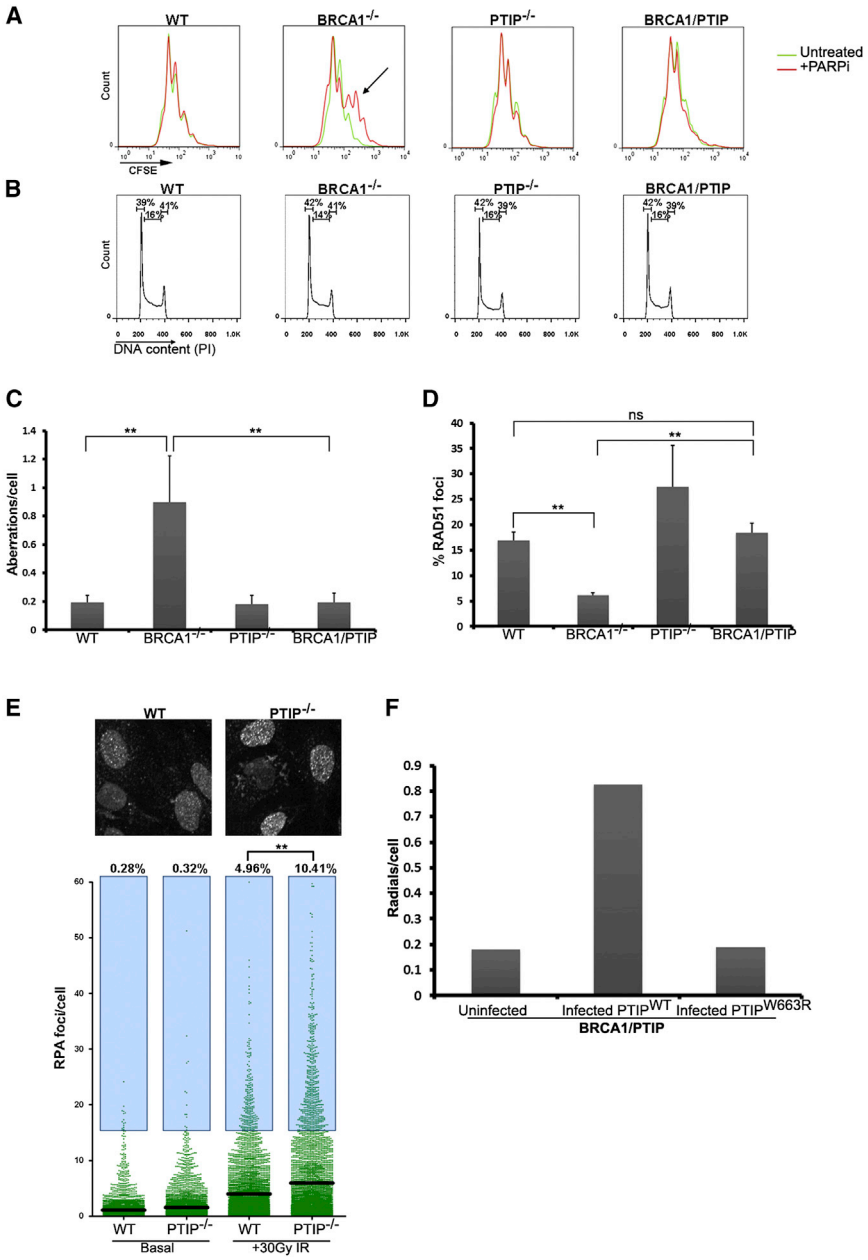

Figure 5. Ablation of PTIP Rescues Homologous Recombination in BRCA1-Deficient Cells

(A) WT, *BRCA1*$^{-/-}$, *PTIP*$^{-/-}$, and *BRCA1*$^{-/-}$ *PTIP*$^{-/-}$ B cells were pulsed with CFSE and stimulated with (red) or without (green) PARPi. CFSE signal diminishes with increasing division. *BRCA1*$^{-/-}$ cells are sensitive to PARPi (arrow indicates sluggish cells) but loss of PTIP in BRCA1-deficient cells rescues the proliferation defect.

(B) WT, *BRCA1*$^{-/-}$, *PTIP*$^{-/-}$, and *BRCA1*$^{-/-}$ *PTIP*$^{-/-}$ B cells were stimulated with LPS+IL4 and cell-cycle distribution was monitored by propidum iodide (PI) staining. Percentage of cells in G1, S, and G2/M is indicated.

(C) Analysis of genomic instability (radial chromosomes, chromatid breaks, and chromosome breaks) in metaphases from B cells treated with 1 μM PARPi. At least 50 metaphases were analyzed for each genotype.

(D) B cells were stimulated for 2 days, irradiated with 10 Gy, and the percentage of cells with immunofluorescent RAD51 foci were quantified (at least 400 cells counted for each genotype).

Data in (B) and (C) represent mean of three experiments ± standard deviations. **p < 0.05 (two-tailed unpaired t test), ns, not significant.

(E) High-throughput microscopy quantification of RPA foci per cell in WT and *PTIP*$^{-/-}$ MEFs that were either untreated or treated with 30 Gy IR. Top: representative image of chromatin bound RPA in irradiated WT and *PTIP*$^{-/-}$ cells. Bottom: quantitation of RPA foci. Bar indicates the mean number of RPA foci per cell, and the blue box designates cells with more than 15 foci, whose percentage is indicated above each box. **p < 0.001.

(F) *BRCA1*$^{-/-}$*PTIP*$^{-/-}$ B cells were reconstituted with PTIP^{WT} or PTIP^{W663R} retroviruses (expressing a GFP marker driven by an internal ribosome entry site) and treated with PARPi. Cells were sorted (GFP^{positive} = infected and GFP^{negative} = uninfected) and metaphases were analyzed for radial chromosomes (n = 50 metaphases analyzed in each case).

See also Figures S3 and S4.

without compromising B cell immunoglobulin class switching by inhibiting the recruitment of PTIP to DSBs.

EXPERIMENTAL PROCEDURES

Mice, MEFs, B Cell Culture, and Infections

53BP1$^{-/-}$ (Ward et al., 2004), *BRCA1*$^{f(\Delta 11)/f(\Delta 11)}$ (NCI mouse repository), *RIF1*$^{f/f}$ (Buonomo et al., 2009; Di Virgilio et al., 2013), and *PTIP*$^{f/f}$ (Daniel et al., 2010) mice have been described. Resting splenic B cells were isolated from 8- to 12-week-old WT or mutant spleen with anti-CD43 microbeads (anti-Ly48; Miltenyi Biotech) and were cultured with LPS (25 μg/ml; Sigma) and IL-4 (5 ng/ml; Sigma) or αCD40 (1 μg/ml; eBiosciences) and IL4 as described (Barlow et al., 2013; Wesemann et al., 2011). WT, 53BP1$^{-/-}$, and ATM$^{-/-}$ MEFs were immortalized by SV40. SV40T immortalized *PTIP*$^{f/f}$ (Cho et al., 2009) and *RIF1*$^{f/f}$ MEFs were infected with CRE viruses to delete PTIP and RIF1, respectively. PMX-PIE-based retroviruses encoding 53BP1^{DB} and 53BP1^{8A} were previously described (Bothmer et al., 2011). Coding sequences

(D) Quantitation of telomeric fusion frequencies. At least 1,800 chromosomes from each genotype were analyzed. Mean value derived from three independent experiments. **p < 0.01 (two-tailed unpaired t test). Error bars represent SEM.

(E) Distribution of telomeric fusions per metaphase in WT and *PTIP*$^{-/-}$ MEFs. At least 30 cells were examined in each of three independent experiments. p(chi-square) < 1 × 10^{-5}. Error bars represent SEM.

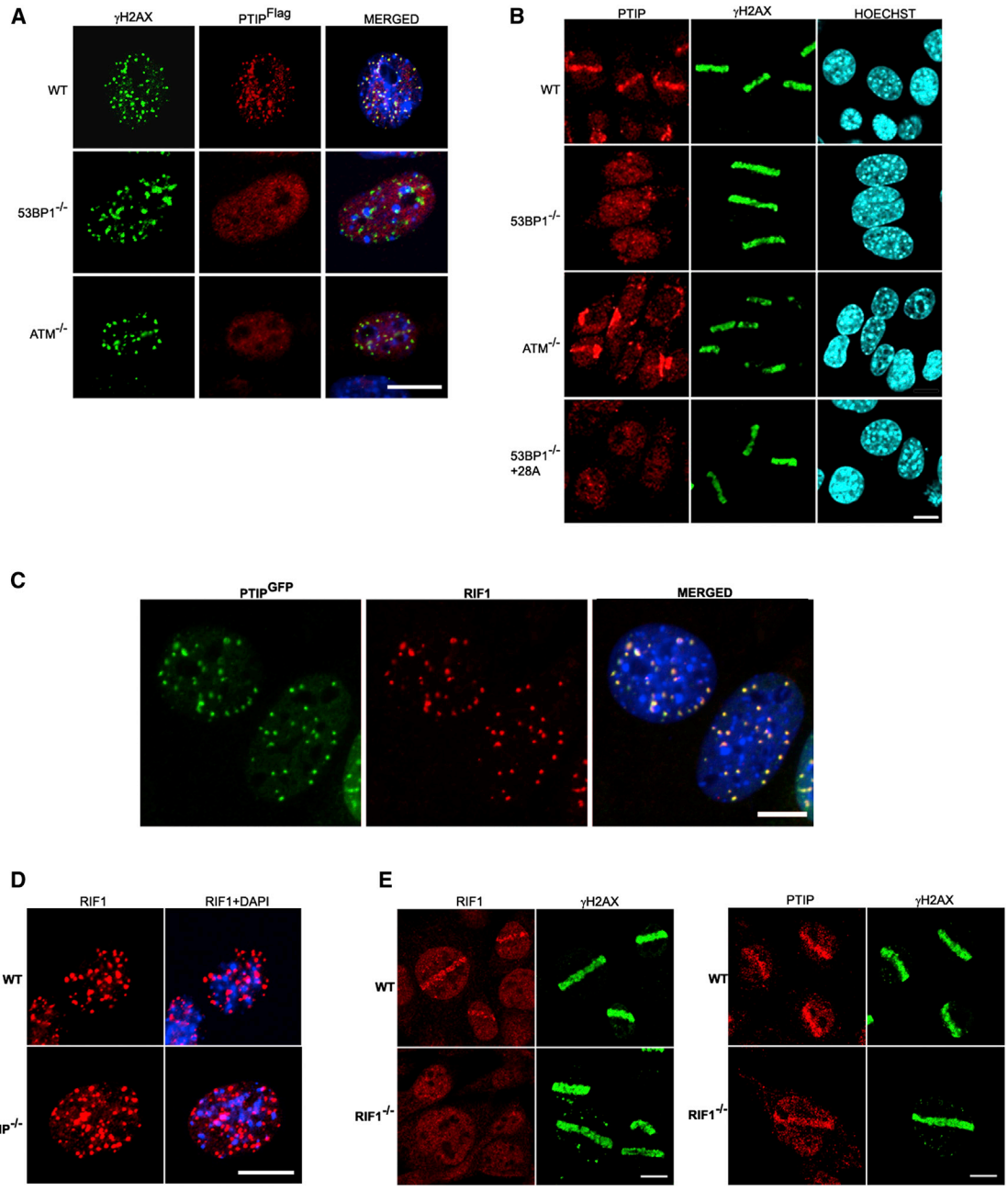

Figure 6. Recruitment of PTIP to DSBs Is ATM and Phospho-53BP1-Dependent but RIF1-Independent

(A) WT, *53BP1*$^{-/-}$, and *ATM*$^{-/-}$ MEFs were infected with a FLAG-tagged WT PTIP retrovirus. Cells were irradiated with 10 Gy, and FLAG (red) IRIF together with γ-H2AX (green) were assessed 4 hr post-IR. DAPI is indicated in blue.

(B) WT, *53BP1*$^{-/-}$, *ATM*$^{-/-}$, and *53BP1*$^{-/-}$ MEFs reconstituted with 53BP1^{28A} were treated with Hoecsht 33342 and then irradiated with a 364 nm laser line. Cells were allowed to recover for 15 min before processing for immunfluorescence analysis of PTIP and γ-H2AX. Hoechst counterstain is indicated in blue.

(C) Cells expressing GFP-PTIP were irradiated with 10 Gy, and PTIP^{GFP} (green) and RIF1 (red) IRIF were assessed 4 hr later. A representative image is shown; 82% of PTIP IRIF colocalized with RIF1 foci and 78% of RIF1 colocalized with PTIP foci (n ≥ 800 foci examined; cells had on average 28 foci).

(D) RIF1 IRIF (red) in irradiated WT and *PTIP*$^{-/-}$ MEFs.

(E) RIF1 (red) and PTIP (red) recruitment to laser scissors damage in WT and *RIF1*$^{-/-}$ MEFs. Damaged cells are indicated by γ-H2AX tracks (green). Scale bars, 10 μm.

See also Figure S5.

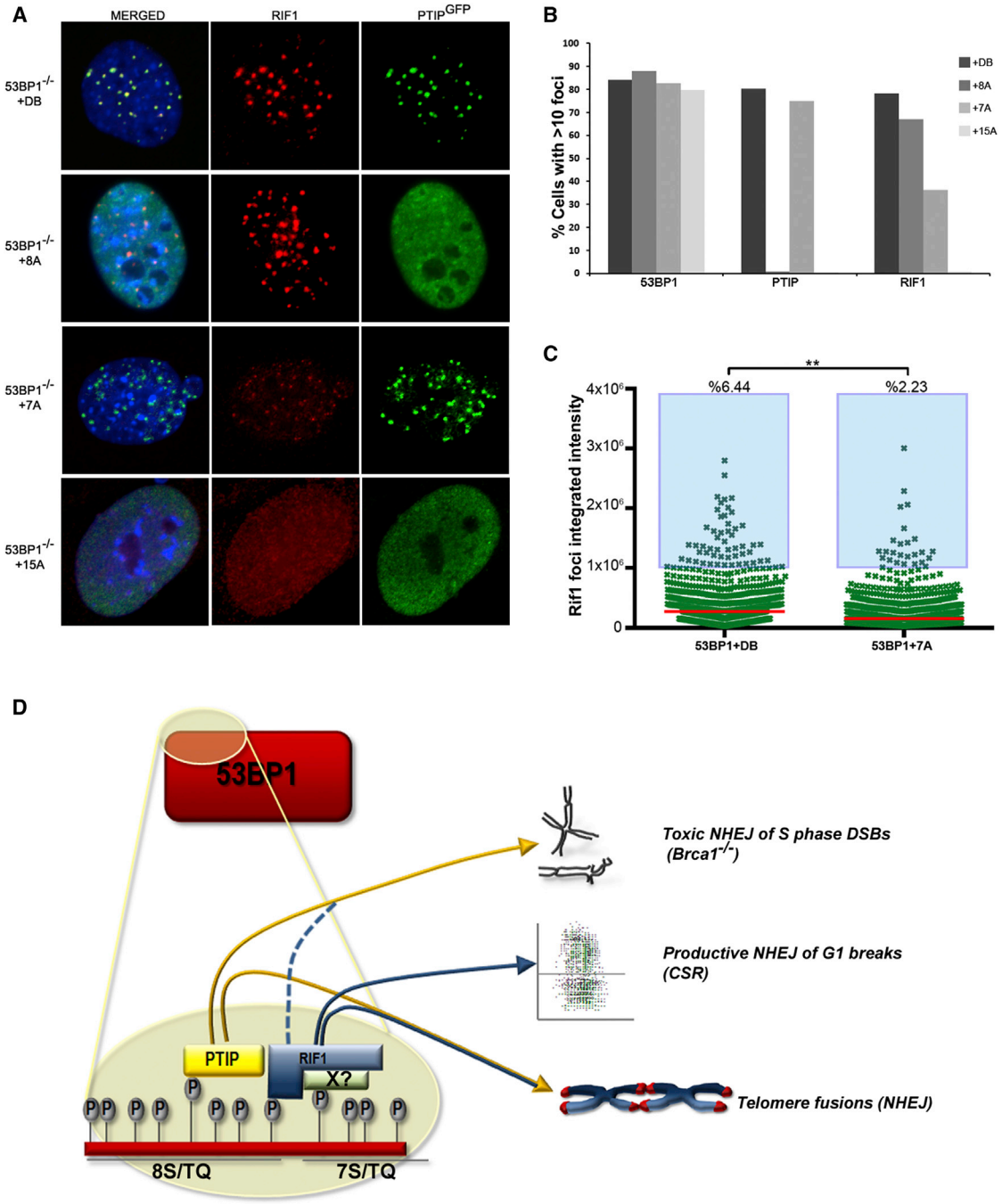

Figure 7. PTIP and RIF1 Association with DSBs Is Dependent on Distinct Phosphorylation Sites on 53BP1

(A) *53BP1*$^{-/-}$ MEFs (reconstituted with 53BP1^{DB}, 53BP1^{8A}, 53BP1^{7A}, or 53BP1^{15A}) were costained with RIF1 (red) and PTIP (green).

(B) Quantitation of percent 53BP1^{DB}, 53BP1^{8A}, 53BP1^{7A}, or 53BP1^{15A} cells with greater than ten 53BP1, PTIP, or RIF1 foci. At least 100 cells were analyzed for each genotype.

(C) Integrated intensity of individual RIF1 IRIF in *53BP1*$^{-/-}$ MEFs reconstituted with DB or 7A. Average RIF1 foci intensity (red line) is 1.6-fold greater in DB versus 7A (**$p < 0.001$, one-tailed unpaired t test), and a greater percentage of very intense foci (z score > 3) are generated in 53BP1^{DB} compared to 53BP1^{7A} (blue box).

(D) Model for regulation of 53BP1 pro-NHEJ and anti-HR activities by distinct phosphointeractions with RIF1 and PTIP, respectively. PTIP binds to the 8S/TQ sites. RIF1 recruitment is largely dependent on C-terminal 7S/TQ sites, but RIF1 may also be stabilized by interactions with 8S/TQ. An unknown factor (X) may bind directly to phosphorylated 53BP1 and mediate RIF1 recruitment, whereas PTIP interaction with 53BP1 is direct (Munoz et al., 2007).

See also Figures S6 and S7.

for mouse PTIP^{WT}/PTIP^{W663R} and PTIP-GFP were cloned into the PMX-IRES-GFP and MIG-IRES-mCherry retroviral vectors, respectively. PARP (KU58948), ATM (Ku55933), and DNA-PK (NU7026) inhibitors were obtained from Astra Zeneca and ATRi has recently been described (Toledo et al., 2011).

Flow Cytometry, Metaphase Analysis, and Telomere FISH

For FACs analysis, splenic B cells were stained with fluorochrome-conjugated anti-B220, anti-igG1, and anti-igE antibodies (PharMingen) as described (Wesemann et al., 2011). Carboxyfluorescein succinimidyl esther (CFSE) labeling was performed to track cell division. Samples were acquired on a FACSCalibur (Becton Dickinson), and cell sorting was preformed on a FACsAria (Becton Dickinson). Cells were harvested for metaphase analysis as described (Callén et al., 2007a). The murine TRF2 shRNA-targeting construct and MEF retroviral infection have been described (Rai et al., 2010). Telomere-induced foci were visualized by hybrization with anti-mouse γ-H2AX antibody (Upstate Biotechnology) together with PNA probe (Applied Biosystems). Phosphorylated Kap-1 was detected by flow cytometry after intracellular staining using the BD Cytofix/Cytoperm kit (BD Biosciences).

DNA Damage, Laser Microirradiation, Immunoprecipitation, and RNA-Sequencing

Cells were treated with different DNA damaging agents (IR, CPT, CisPt, and PARPi), and colony survival was assessed after 14 days, or metaphase analysis was performed 24 hr after treatment. For immunofluorescent staining, cells were irradiated with indicated doses of ionizing radiation, allowed to recover, and then fixed and processed as described (Celeste et al., 2003). For microirradiation, cells were presensitized in DMEM media containing 0.1 μg/ml of Hoechst 33342 for 60 min before replacing with phenol red free media containing 5 mM HEPES, and then irradiated with the 364 nm laser line on a LSM510 confocal microscope (Zeiss) equipped with a heated stage. Cells were allowed to recover for 15 min prior to processing for immunofluorescence. Analysis of RPA foci was performed using an Opera High-Content Screening system as described (López-Contreras et al., 2012). Primary antibodies for immunofluorescence were rabbit anti-53BP1 (Novus), mouse anti-γ-H2AX (Upstate Biotechnology), mouse or rabbit anti-FLAG-M2 (Sigma), mouse anti-AIM1 (Becton Dickinson), mouse anti-GFP (Roche), rabbit anti-RAD51 (Santa Cruz), rat anti-RPA (Cell Signaling), rabbit-anti-PTIP (Cho et al., 2009), and rabbit-anti-RIF1(Di Virgilio et al., 2013). DNA was counterstained with DAPI. For immunoprecipitation, primary *53BP1*$^{-/-}$ B cells were infected with retroviral constructs. Ninety-six hours postactivation, cells were irradiated (10 Gy), left to recover for 45 min, and collected by centrifugation. Cells were lysed, sonicated, and cell lysates were incubated with magnetic beads (M-270 epoxy beads, Invitrogen) conjugated with anti-Flag M2 antibody (Di Virgilio et al., 2013). 53BP1-associated proteins were eluted by incubation in NuPAGE LDS sample buffer (Invitrogen) supplemented with 45 mM DTT for 10 min at 72°C. For RNA sequencing, reads from each cDNA library were mapped onto the Build 37 assembly of the National Center for Biotechnology Information mouse genome data (July 2007; NCBI37/mm9) using TopHat. Bioconductor (Gentleman et al., 2004) was used to calculate the RPKM (reads per kilobase exon model per million mapped reads) of the RefSeq annotated genes.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.05.023.

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Molecular Cell
Review

The AID-Induced DNA Damage Response in Chromatin

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Chemical modifications to the DNA and histone protein components of chromatin can modulate gene expression and genome stability. Understanding the physiological impact of changes in chromatin structure remains an important question in biology. As one example, in order to generate antibody diversity with somatic hypermutation and class switch recombination, chromatin must be made accessible for activation-induced cytidine deaminase (AID)-mediated deamination of cytosines in DNA. These lesions are recognized and removed by various DNA repair pathways but, if not handled properly, can lead to formation of oncogenic chromosomal translocations. In this review, we focus the discussion on how chromatin-modifying activities and -binding proteins contribute to the native chromatin environment in which AID-induced DNA damage is targeted and repaired. Outstanding questions remain regarding the direct roles of histone posttranslational modifications and the significance of AID function outside of antibody diversity.

Introduction

Chromatin is the platform for transcription, DNA repair, and recombination. Posttranslational modifications (PTMs) of the histone H3, H2B, H2A, and H4 components of chromatin regulate DNA-mediated processes by altering chromatin structure and generating recognition sites for mediating effector protein stabilization (Downs et al., 2007; Felsenfeld and Groudine, 2003; Jenuwein and Allis, 2001; Suganuma and Workman, 2011). The histone code hypothesis that a particular histone PTM, or combination thereof, can constitute a code for a cellular action or biological function continues to be tested and has permeated far across the field of DNA repair from its inception in relation to gene regulation (Downs et al., 2007; Jenuwein and Allis, 2001). The first and still most striking and clear example of how a particular histone modification promotes genome stability came from the observations that mice deficient in the histone variant H2AX, which becomes phosphorylated at serine 139 s after ionizing radiation (IR)-induced DNA damage (Bonner et al., 2008), accumulate spontaneous DNA double-strand breaks (DSBs) and develop tumors more rapidly when cell-cycle checkpoints are compromised (Bassing et al., 2003; Celeste et al., 2003). In this review, we describe our current understanding of how histone PTMs function in a physiological setting, with immunoglobulin class switch recombination (CSR) as a model. The CSR reaction at the immunolglobulin heavy-chain (*Igh*) locus can be divided into three general temporal stages, including the so-called germline transcription step that mediates chromatin accessibility, the targeting and generation of activation-induced cytidine deaminase (AID)-induced DNA damage, and the subsequent repair of CSR-associated DSBs (Figure 1). The two main focus points of this review are to discuss our understanding of how chromatin PTMs function in the accessibility phase as well as in the repair phase of AID-induced DNA damage.

Initiating the Second Wave of Antibody Diversity with AID

B lymphocytes undergo physiological DNA damage to produce large numbers of antibody molecules that are poised to associate with foreign antigens. This antigen/antibody interaction will subsequently activate pathways for the removal and clearance of pathogens from our body as part of the adaptive immune response. Each B lymphocyte expresses a single B cell receptor (BCR) that has been assembled by RAG1/RAG2-dependent V(D)J recombination during early B cell development in the bone marrow. When mature naive IgM-expressing B cells in peripheral lymphoid organs recognize antigen through their BCR and become activated within germinal center structures, they undergo clonal expansion and further diversify their antigen receptors with somatic hypermutation (SHM) and isotype class switching. The introduction of mutations in rapidly proliferating B cells during SHM ultimately culminates in the production of thousands of B cells expressing slightly different receptors with varying specificity for an antigen, from which the B cell with the highest affinity for the antigen can be selected. Isotype class switching alters antibody effector function, whereby activated B cells swap constant region gene segments of the antibody without altering variable region specificity. This CSR event requires DNA DSB formation and DNA end-joining (Figure 1). Expression of a successfully recombined class-switched *Igh* gene can help eliminate particular pathogens by activating, for example, phagocytic immune cells (Boboila et al., 2012; Stavnezer et al., 2008).

AID has taken center stage as a B cell-specific factor required for both SHM and CSR. It was first identified as being differentially expressed in a murine B lymphocyte cell line after stimulation to undergo antibody class switching and was shown to be highly expressed in primary human and murine germinal center B cells from tonsil, lymph node, and spleen (Muramatsu et al., 2007). In addition to expression in germinal center B cells,

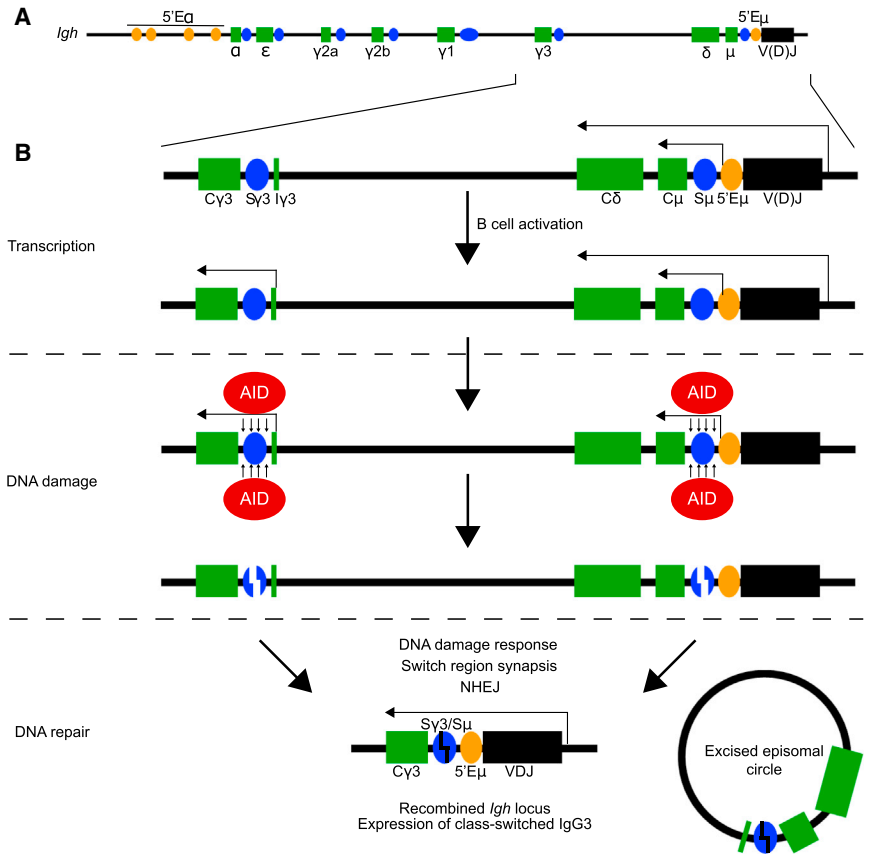

Figure 1. The Transcription, DNA Damage, and DNA Repair Phases of CSR

(A) Organization of the *Igh* locus in mice, including the antigen recognition V(D)J gene segment in black, the switch (S) regions in blue, the constant (C) region exon segments in green, and the enhancers in orange. The μ, δ, γ3, γ1, γ2b, γ2a, ε, and α isotypes correspond to immunoglobulins M, D, G3, G1, G2b, G2a, E, and A.

(B) In resting B cells, transcription initiating upstream of the V(D)J gene segment leads to a full-length *Igh* transcript and initiates from 5′ Eμ to generate the μ germline noncoding sterile switch transcript. However, transcription is completely absent from the downstream cluster of switch regions and constant gene segments. Upon B cell activation during an immune response (or by LPS stimulation in cell culture), germline transcription initiates from a switch promoter region. Accessibility of AID to transcribed switch region chromatin targets AID activity to *Igh*, leading to DSB formation. Synapsis of the two broken switch regions and their repair, mediated by the DNA damage response and NHEJ machineries, promotes efficient CSR (to IgG3 in this example) and suppresses genomic instability.

there are reports that AID is expressed in oocytes and, albeit at far lower levels, in embryonic stem cells, early embryos, primordial germ cells, testes, and B cell progenitors (Orthwein and Di Noia, 2012). Mutations in the gene encoding AID, *Aicda*, were found to cause the rare autosomal recessive hyper-IgM syndrome type 2 (HIGM2) that is characterized by the absence of immunoglobulin CSR and somatic hypermutation (Revy et al., 2000). Subsequently, AID$^{-/-}$ mice recapitulated this B cell disease without showing any other noticeable developmental phenotypes (Muramatsu et al., 2000). Interestingly, in an artificial system in which AID is not normally present, expression of AID and a class-switching DNA substrate was shown to be sufficient for CSR in fibroblasts, suggesting that AID may be the sole B cell-specific factor required for initiating CSR (Muramatsu et al., 2007).

During an immune response, peripheral B cells stimulated by antigen and the cytokine milieu become activated and enter the cell cycle; shortly thereafter, the cells begin to express AID and concentrate in germinal center structures of lymph nodes and the spleen (Victora and Nussenzweig, 2012). For SHM, AID is targeted to the variable V(D)J gene segments of the immunoglobulin heavy and light chains and catalyzes cytosine deamination events that lead to increased mutations as a result of error-prone translesion DNA polymerase activity and DNA replication (Di Noia and Neuberger, 2007; Liu and Schatz, 2009). To initiate the CSR reaction, AID gets targeted to the *Igh* locus downstream of the V(D)J gene segment and initiates DNA lesions that, here, lead to DNA DSB formation (Boboila et al., 2012; Nussenzweig and Nussenzweig, 2010; Stavnezer et al., 2008). For productive CSR, AID-induced DSBs must occur at two switch (S) repeat regions (i.e., Sμ, Sγ3, Sγ1, Sγ2b, Sγ2a, Sε, or Sα in the mouse) that precede participating constant region gene segments (Stavnezer et al., 2008) (Figure 1). Synapsis and DNA repair of the two broken DNA ends are then mediated by protein factors of the DNA damage response (DDR) and the nonhomologous end-joining (NHEJ) pathways. This DNA rearrangement process resulting in an orchestrated switch from IgM expression to expression of IgG, IgE, or IgA must be carefully controlled and coordinated in the context of chromatin.

The Accessibility Hypothesis for AID Targeting

The lineage specificity of CSR can be explained by the fact that AID expression is largely restricted to germinal center B cells. To explain how DNA rearrangements in lymphocytes occur specifically at antigen receptor gene loci, Yancopoulos and Alt (1985) put forth the accessibility hypothesis after observing sterile germline transcript initiation only at gene segments undergoing recombination. Since then, germline transcripts coinciding with recombination at a particular gene segment have been observed at all antigen receptor loci, and many lines of evidence now support the conclusion that germline transcription of an antigen receptor gene segment is an essential feature of the targeting mechanism for both RAG1/RAG2-mediated V(D)J recombination in early developing lymphocytes (Cobb et al., 2006; Krangel, 2009) and AID-dependent CSR and SHM in mature B cells (Stavnezer et al., 2008). In addition to germline expression of sterile noncoding transcripts, the accessibility hypothesis has expanded to encompass the spatial organization

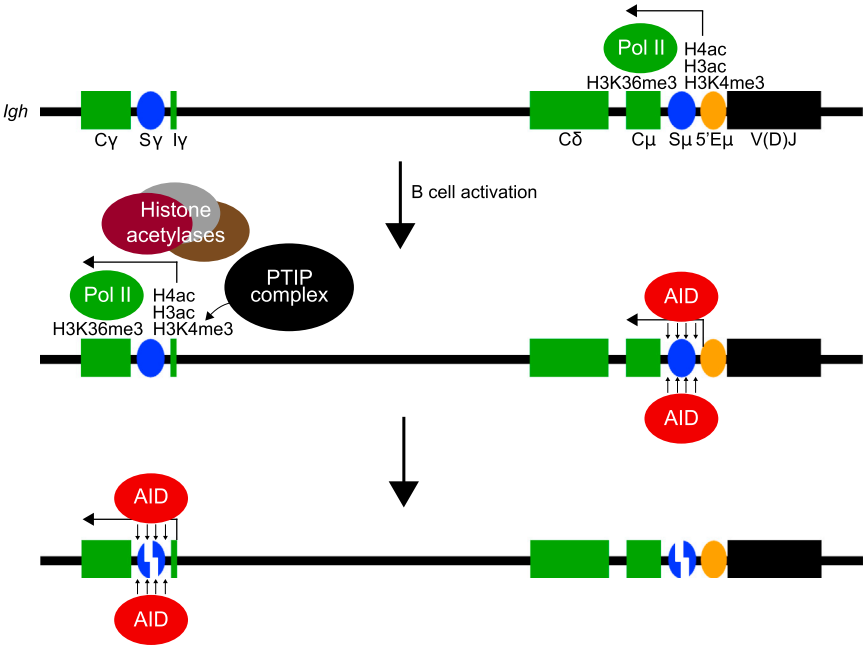

Figure 2. Transcription-Linked Histone PTMs and PTIP Promote Accessibility for AID Targeting

PTMs of active chromatin are found at the μ region of *Igh* in resting B cells and help to establish a chromatin environment that is permissive for AID targeting. Upon B cell activation, transcription-linked histone PTMs become detectable at downstream switch regions that have been induced to undergo CSR. These inducible histone PTMs at downstream switch regions are dependent on PTIP, at least at the γ3, γ1, and γ2ab regions.

and nuclear positioning of antigen receptor loci (Alt et al., 2013; Hewitt et al., 2010; Jhunjhunwala et al., 2009; Johnson et al., 2009). For the purposes of our discussion, we define accessibility as a localized alteration of chromatin structure that facilitates recombination at the locus, and we focus our discussion on how germline transcription and histone modifications target AID for CSR.

The importance of transcription in targeting the somatic hypermutation activity first came from a genetics experiment showing that insertion of the variable region promoter at a location upstream of the constant region at the Igκ locus promoted hypermutation at the constant region, where it normally does not occur (Peters and Storb, 1996). At the *Igh* locus, all germline switch transcripts have the same overall structure, with an intronic (I) promoter exon followed by a switch (S) region and a constant (C) region gene segment (Stavnezer et al., 2008) (Figure 2). Activation signals during an immune response direct promoter-driven germline transcription and DSB formation to particular switch regions at the *Igh* locus (Boboila et al., 2012; Stavnezer et al., 2008). Indeed, genetic studies in mice revealed that deletion of Iγ1 abolished CSR to IgG1 (Jung et al., 1993) and that replacement of the Iγ2b promoter and I exon with a neomycin-resistance gene transcribed in the antisense direction abolished CSR to IgG2b (Zhang et al., 1993). The conserved structure of the germline transcripts between the different immunoglobulin isotypes suggests that these noncoding RNAs may have a common function in CSR, and elegant mouse genetic studies have suggested that splicing of the I and C exons is required for CSR (Harriman et al., 1996; Hein et al., 1998; Lorenz et al., 1995); nevertheless, the role of the germline transcripts and their splicing is still poorly understood.

The mechanism for targeting AID to immunoglobulin loci is of great interest, given its role in inducing DNA mutations and tumorigenesis (Di Noia and Neuberger, 2007; Pavri and Nussenzweig, 2011; Vuong and Chaudhuri, 2012). Germline transcription of immunoglobulin genes paves the way for AID to act on single-stranded DNA (ssDNA), such that AID can induce mutations on both the template and nontemplate strands, as evidenced by sequencing genomic DNA from B cells undergoing SHM and CSR (Liu and Schatz, 2009; Petersen et al., 2001). The C terminus of AID appears to be required for CSR and the N terminus of AID is required for SHM, suggesting that specific interactions may modulate the targeting and/or function of AID (Di Noia and Neuberger, 2007; Xu et al., 2012). In B cells stimulated to undergo CSR, AID physically associates with RNA polymerase II (RNA Pol II) (Nambu et al., 2003). Under similar conditions, RNA Pol II localizes at *Igh* in a special way, accumulating from the intronic promoter, through the switch region, and into the constant region for both Sμ and the downstream Sγ3 switch region (Daniel et al., 2010; Rajagopal et al., 2009; Wang et al., 2009a). This 5′ end buildup of RNA Pol II at switch regions is independent of AID (Rajagopal et al., 2009; Wang et al., 2009a) and suggests that RNA Pol II stalling may be an intrinsic feature of transcription through the switch regions.

Recent advances have provided additional evidence for how AID activity is linked to transcription by showing that AID directly interacts with Spt5, the RNA exosome, and the polymerase-associated factor (PAF) elongation complex. RNAi knockdown of any of these factors in the CH12 B cell line leads to reduced chromatin association of AID to *Igh* and impairs CSR without affecting germline transcription at the switch region (Basu et al., 2011; Pavri and Nussenzweig, 2011; Stanlie et al., 2012; Willmann et al., 2012). Together, these studies lead to a model whereby RNA Pol II stalling facilitates AID recruitment through Spt5 and the RNA exosome, which promotes subsequent targeting of mutations on both DNA strands (Basu et al., 2011; Pavri and Nussenzweig, 2011; Willmann et al., 2012; Yamane et al., 2011). Thus, the process of transcription, or even the transcript itself, may play a direct role in AID targeting, and the function of germline transcription may not be limited to simply rendering the target DNA accessible to AID.

Correlating Histone Modifications with AID Localization

Alterations in chromatin structure that are specific consequences of germline transcription at *Igh* may also play a role in promoting and/or stabilizing AID targeting. Using in vitro systems, AID can efficiently target DNA in nucleosomes undergoing transcription, and there is evidence that nucleosome stability and positioning can significantly influence AID targeting (Kodgire et al., 2012; Shen et al., 2009). Looking more closely at particular aspects of chromatin structure, multiple studies have correlated localized changes in histone modifications with accessibility for antigen receptor gene recombination. Histone marks including acetylation of H3 and H4 occur at the *Igh* switch regions in B cells actively undergoing CSR (Chowdhury et al., 2008; Daniel et al., 2010; Kuang et al., 2009; Li et al., 2004; Nambu et al., 2003; Wang et al., 2006, 2009a), but the most clear and direct link has come from the interaction between H3K4me3 and RAG1/RAG2 during V(D)J recombination. The transcription initiation-associated histone H3K4me3 mark (Ruthenburg et al., 2007) can be directly recognized by a plant homeodomain (PHD) finger in RAG2, and this interaction appears to help target and stimulate RAG1/RAG2 activity (Schatz and Swanson, 2011). Indeed, decreases in H3K4me3 result in defective RAG1/RAG2-mediated recombination (Matthews et al., 2007).

Additional clues about the relation between histone marks and accessibility during CSR come from comparison of histone modification profiles of resting and stimulated B cells. For example, the chromatin structure at Sμ that is present in resting B cells is sufficient to target AID activity. This was shown by measuring AID-induced mutations at Sμ in resting B cells from mice constitutively expressing AID when it is not normally present in B cell progenitors (Robbiani et al., 2009) (Figure 2). These data are consistent with H3K4me3 and the transcription elongation-associated H3K36me3 mark at Sμ being detectable and remaining unchanged after stimulation to undergo CSR (Balter et al., 2012; Daniel et al., 2010; Dayal et al., 2011; Wang et al., 2009a). Furthermore, a study from mice lacking the Sμ tandem repeat sequences showed that a shift in H3K4me3 and H3 acetylation patterns correlated with accessibility to the switch regions and suggested that chromatin accessibility is not strictly dependent on the underlying DNA sequence but instead may be controlled by a combination of promoter location, the extent of RNA Pol II association, and histone modifications (Balter et al., 2012; Min et al., 2005).

In contrast to Sμ, H3K4me3, H3K36me3, and RNA Pol II are found at the downstream *Igh* switch regions only after B cell stimulation (Daniel et al., 2010; Wang et al., 2009a). In activated B cells, these histone marks at the *Igh* locus are independent of AID (Daniel et al., 2010; Wang et al., 2009a), consistent with their association with transcription rather than with DNA DSBs (Ruthenburg et al., 2007). One particular feature of H3K4me3 at Sμ and the downstream switch regions is that the peak is localized from the promoter-proximal initiator exon to the end of the switch region; however, it does not accumulate as significantly as RNA Pol II (Daniel et al., 2010; Wang et al., 2009a). This is in contrast to the localization pattern that genome-wide studies have shown for H3K4me3, with the profile generally restricted to within 2 kb of the transcription start site (TSS) (Wang et al., 2009b). Indeed, the peak of H3K4me3 at *Igh*-γ3 is among the broadest of all H3K4me3 peaks in lipopolysaccharide (LPS)-stimulated B cells demarcating near 7 kb of DNA (Daniel et al., 2010).

Analogous to the RAG2/H3K4me3 colocalization at many sites in the genome of developing lymphocytes undergoing V(D)J recombination (Schatz and Swanson, 2011), AID surprisingly also localizes at many sites in the genome of activated mature B cells, as demonstrated by chromatin immunoprecipitation sequencing (ChIP-seq) analyses (Yamane et al., 2011). In this report, AID was found to associate with chromatin in the vicinity of nearly 6,000 genes, raising questions as to whether AID has off-target activity or additional functions beyond antibody diversification. Interestingly, histone acetylation and H3K4me marks were significantly enriched at sites of AID localization, as was RNA Pol II and transcriptional activity (Pavri and Nussenzweig, 2011; Yamane et al., 2011). There is also evidence from other groups that SUV39H1-mediated H3K9me3, which normally is associated with gene repression, may function in AID targeting to the *Igh* locus (Bradley et al., 2006; Chowdhury et al., 2008; Jeevan-Raj et al., 2011; Kuang et al., 2009). Nevertheless, as demonstrated by the widespread AID-induced DNA damage in mice lacking the chromatin-associated DDR factor 53BP1 (as discussed later in the review) (Klein et al., 2011; Yamane et al., 2011), AID activity correlates with an accessible chromatin configuration.

Chromatin-Modifying Activities Promoting Accessibility for AID

One insight into how chromatin-modifying activities may promote accessibility for AID stems from the study of the Pax transactivation domain-interacting protein (PTIP), which harbors six BRCA1 C-terminal (BRCT) domains and is implicated in both gene expression and the DDR (Daniel and Nussenzweig, 2012; Muñoz and Rouse, 2009). The PTIP component of a mixed-lineage leukemia (MLL)-like H3K4 methyltransferase complex was shown to be critical for promoting H3K4me3, histone acetylation, and germline transcription at *Igh* switch regions, leading to CSR of multiple isotypes (Daniel et al., 2010). Specifically, primary B cells from B lymphocyte-specific PTIP conditional deletion mice displayed defects in IgG3, IgG2b, and IgG1 class switching, concomitant with loss of RNA Pol II association and transcription initiation at the γ3, γ2b, and γ1 downstream switch regions, respectively (Daniel et al., 2010). As PTIP stably associates with a subset of MLL-like complexes that contain the MLL3/KMT2C and MLL4/KMT2B methyltransferases and display activity for histone H3K4 (Muñoz and Rouse, 2009), the study established that specific chromatin changes may in fact control the accessibility of the *Igh* locus for CSR (Daniel et al., 2010; Schwab et al., 2011). Moreover, it was recently shown that PTIP also controls accessibility of the T cell receptor α locus during V(D)J recombination (Callen et al., 2012). In both cases, it remains to be determined whether H3K4me3 precedes transcription or vice versa. Thus, the clustered, highly repetitive, and tightly repressed gene segments within the *Igh* and *Tcra* loci, which normally lack RNA Pol II and H3K4me3, require PTIP to promote the necessary DSB targeting of the locus.

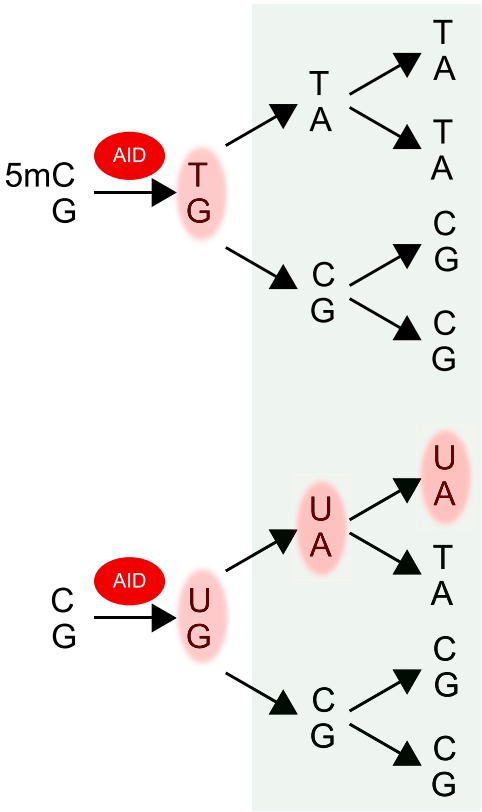

Figure 3. Mutagenic Consequences of AID-Mediated Deamination of Cytosine and 5mC if Not Excised during Subsequent Cell Cycles
AID catalyzes the deamination of cytosine nearly 10-fold higher than 5mC. Deamination events catalyzed by AID would normally be recognized and excised by the BER and/or MMR machinery and either repaired in an error-free manner, repaired in an error-prone manner with translesion polymerases, or left unrepaired as a single-strand break. Base pairs shaded in pink are mispaired and would be recognized by repair machineries. Shaded in green, to the right, are the consequences of an initial DNA lesion that failed to be excised and was transmitted to daughter cells. Note that the consequences are nearly identical.

A different study using the CH12 B cell line provided genetic evidence, using RNAi knockdown, that MLL-like methyltransferase activities are dispensable for *Igh* germline transcription at the α switch region but required for H3K4me3, DSB formation, and CSR at the Sα (Begum et al., 2012; Stanlie et al., 2010). From this study, H3K4me3 was proposed to serve as a mark for recruiting the recombinase machinery for CSR independently of its function in transcription (Stanlie et al., 2010). Thus, further investigation is needed to clarify the role(s) of H3K4me3 in CSR.

Processing of AID-Induced DNA Mutations and DSB Formation

As a cytosine deaminase, multiple laboratories have established that AID acts on ssDNA and has an activity about 8-fold higher for cytosine compared to 5-methylcytosine (5mC), with no activity for 5-hydroxymethylcytosine (5hmC) (Di Noia and Neuberger, 2007; Franchini et al., 2012; Nabel et al., 2012). As an epigenetic mark on DNA, as opposed to histones, 5mC accounts for about 4% of cytosine bases in the mammalian genome and typically occurs as CpG dinucleotides that can stably silence expression of a gene (Law and Jacobsen, 2010). A potential role for AID in DNA demethylation has been proposed, although the biological significance of this activity for B lymphocytes or other cell types remains to be established (Fritz and Papavasiliou, 2010). Furthermore, it remains unclear how methylated CpG motifs in genomic DNA might be sufficiently targeted by deaminases that prefer ssDNA. The available data currently support a model in which AID deaminates cytosine bases in DNA to generate uracil and deaminates 5mC at low levels to generate thymine, both of which lead to dT/dA transitions from dC/dG (Figure 3).

Great progress has been made in understanding the mechanisms explaining how AID-mediated cytosine deamination leads to SHM or CSR (Di Noia and Neuberger, 2007). Powerful genetic evidence for the role of the base excision repair (BER) machinery in the processing of AID-induced DNA lesions stems from the identification of mutations in the gene encoding uracil DNA glycosylase, *UNG*, from a subset of hyper-IgM syndrome patients (Imai et al., 2003). Furthermore, genetic ablation of UNG in mice leads to the detection of uracil in the DNA of immunoglobulin genes, a significant increase in transition mutations at dC/dG pairs without affecting dA/dT pairs, and reduced SHM and CSR (Maul et al., 2011; Rada et al., 2002). These data suggest that uracil excision at *Igh*-V and switch regions is inhibited by UNG deficiency and that replication over increased dU/dG lesions leads to dT/dA mutations (Figure 3). Mice and cells deficient in the *Ape1* apurinic/apyrimidinic endonuclease of the BER pathway have also been shown to display reduced CSR, suggesting that abasic sites on opposite strands may cause single-strand breaks that, when sufficiently close, lead to DSBs (Masani et al., 2013; Stavnezer et al., 2008). In addition, there is evidence from mouse models deficient in MSH2, MSH6, MLH1, MLH3, PMS1, PMS2, or EXO1 that the mismatch repair (MMR) machinery also functions to promote CSR and dA/dT mutations during SHM (Di Noia and Neuberger, 2007; Stavnezer et al., 2008).

Overlapping roles for the BER and MMR pathways in generating antibody diversity were demonstrated by showing that a combined deficiency in both pathways, as shown by $Ung^{-/-}Msh2^{-/-}$ and $Ung^{-/-}Msh6^{-/-}$ mice, leads to a complete ablation of CSR and a complete loss of mutations at dA/dT during *Igh*-V SHM (Rada et al., 2004; Shen et al., 2006). The mutation spectra observed in $Ung^{-/-}Msh2^{-/-}$ B cells stimulated ex vivo established that AID targets both strands of DNA subsequent to initiation of cytokine-directed germline transcription at switch regions (Xue et al., 2006). Moreover, while so-called R loop secondary structures of G-rich sequences in the nontemplate strands of the switch repeats may enhance CSR as proposed (Yu et al., 2003), they do not appear to be strictly required for AID targeting since V gene segments mutated during SHM are not GC rich, nor were R loops detected at this region in primary B cells (Pavri and Nussenzweig, 2011). These data support the model in which AID-mediated dU/dG mismatches can cause strand-symmetric mutations either by being replicated over during S phase (Figure 3) or by being substrates for BER and MMR pathways. Abasic sites and stretches of ssDNA caused by EXO1-mediated resection around the abasic site

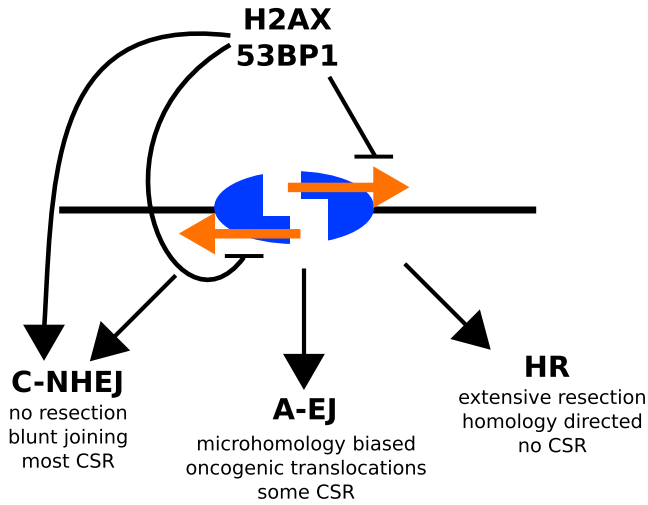

Figure 4. The γH2AX/MDC1 and H4K20me/53BP1 Interactions Suppress DNA End Resection and Promote Repair of CSR-Associated Breaks by NHEJ

Under normal conditions, these chromatin-mediated interactions facilitate synapsis and repair of two different broken switch regions. These CSR joins mediated by the C-NHEJ pathway show little evidence of resected DSB ends and constitute the majority of successful CSR. In the absence of C-NHEJ, A-EJ mediates some level of CSR but also mediates aberrant joining in the form of chromosomal translocations. In the absence of either H2AX or 53BP1, extensive resection occurs on unrepaired DSBs, thereby inhibiting NHEJ pathways and ultimately leading to repair of the DSB by HR without class switching. Orange arrows indicate 5′ to 3′ DNA end resection of a DSB incurred within an *Igh* switch region.

could be repaired by low-fidelity translesion polymerases creating mutations at both dC/dG and dA/dT pairs leading to the observed SHM spectra or cause staggered single-strand breaks within switch regions that lead to the DSBs required for CSR (Stavnezer et al., 2008). DSBs at *Igh* are dependent on AID and generated during the G1 phase of the cell cycle, as demonstrated by immunocytochemistry-FISH (fluorescence in situ hybridization) analyses of γ-H2AX or Nbs1 foci colocalizing with the *Igh* locus in B cells actively undergoing CSR (Petersen et al., 2001). However, since AID is expressed throughout the cell cycle and could presumably generate DNA damage in both G1 and S/G2, it remains unclear why the generation of AID-dependent DSBs is restricted to the G1 phase. Moreover, there is no clear evidence regarding whether chromatin modifications are involved in the processing of AID-induced lesions; for example, neither H2AX nor 53BP1 is required for SHM (Nussenzweig and Nussenzweig, 2010).

Repairing AID-Induced DNA Breaks in the Context of Chromatin

Once an AID-mediated break is generated during CSR, the joining of a DSB from one switch region, for example Sμ, to a DSB within a downstream switch region requires the NHEJ pathway. How directional joining for productive CSR is imposed, instead of nonphysiological inversional rearrangements, remains an open question (Boboila et al., 2012; de Villartay et al., 2003; Nussenzweig and Nussenzweig, 2010). Much of our understanding of the NHEJ pathway stems from its function in V(D)J and CSR. It is now clear that the so-called classical NHEJ pathway (C-NHEJ) involving KU70, KU80, DNAPKcs, Artemis, XRCC4, and DNA ligase IV acts throughout the cell cycle and is essential for completion of V(D)J recombination, as single knockout mice for these factors display complete blocks in early B and T lymphocyte development (Boboila et al., 2012). This pathway also plays a significant role in CSR, although residual class switching is observed with genetic deficiency in any of the C-NHEJ factors (Boboila et al., 2012). These and other observations have revealed the existence of alternative end-joining (A-EJ) mechanisms (Boboila et al., 2012). While the C-NHEJ pathway typically joins two DSBs together with minimal processing, joins formed in the absence of C-NHEJ usually display short microhomologies that may have guided repair of a DSB after resection of the DNA end by 5–25 nucleotides (Figure 4). Further elucidation of these A-EJ mechanisms clearly warrants additional investigation, particularly since A-EJ has been implicated in the formation of oncogenic translocations found in lymphoid cancer (Gostissa et al., 2011; Zhang and Jasin, 2011). Unlike the error-prone NHEJ pathways, an error-free pathway for DNA repair is homologous recombination (HR), which only occurs in the S/G2 phases of the cell cycle when a sister chromatid or homologous chromosome exists to use as a template for repair.

Upon DSB formation, the DDR involves PTMs of many proteins to signal for the repair of the DSB; thus far, these include phosphorylation, methylation, acetylation, ubiquitination, sumoylation, and poly(ADP-ribosyl)ation (Ciccia and Elledge, 2010; Lukas et al., 2011; Polo and Jackson, 2011). For example, the RNF8 and RNF168 ubiquitin ligases initiate a cascade of ubiquitination (at sites of DSBs) that functions, at least in part, to accumulate 53BP1 and BRCA1 to DNA breaks (Jackson and Durocher, 2013). In addition, while poly(ADP)-ribosylation catalyzed by poly(ADP)-ribosylation polymerases (PARPs) functions in single-strand break repair, this PTM may facilitate DSB repair by transcriptional silencing of the chromatin flanking the damaged sites (Lukas et al., 2011). Protein modules that recognize PTMs can help to target a protein to sites of DNA damage. For example, the BRCT and FHA domains found in several DNA repair proteins are phosphoprotein recognition domains (Polo and Jackson, 2011), while the Tudor domain is a methyllysine recognition domain (Daniel et al., 2005).

To date, there are two well-established direct protein interactions important for DNA repair during CSR that involve recognition of histone PTMs. The MDC1 protein contains a BRCT domain that associates with the histone variant H2AX phosphorylated at S139 (γ-H2AX), and the tandem Tudor domains of 53BP1 recognize histone H4K20me1 and H4K20me2 (Downs et al., 2007). γ-H2AX is a hallmark of DSBs, becoming phosphorylated seconds after IR or laser-induced DNA damage (Bonner et al., 2008). As an integral nucleosomal component of chromatin, γ-H2AX functions as a haploinsufficient tumor suppressor, at least in part, by promoting the accumulation of many DDR factors to sites of DNA damage (Bassing et al., 2003; Celeste et al., 2003). Cells and mice lacking H2AX display general and *Igh*-associated genomic instability and a mild defect in CSR (Celeste et al., 2002; Franco et al., 2006). 53BP1 also functions as a haploinsufficient tumor suppressor,

whose disruption causes a profound defect in CSR (Manis et al., 2004; Reina-San-Martin et al., 2007; Ward et al., 2005, 2004), and strikingly rescues HR, PARP inhibitor sensitivity, and early embryonic lethality of *Brca1* nullizygous cells and mice (Bouwman et al., 2010; Bunting et al., 2012, 2010; Cao et al., 2009). A Tudor domain point mutation that disrupts H4K20 methyllysine recognition abrogates chromatin association of 53BP1 and phenocopies complete 53BP1 deletion with respect to all phenotypes tested, including CSR (Bothmer et al., 2011). Accumulation of 53BP1 at sites of DNA damage is dependent on the H2AX/MDC1/RNF8/RNF168-mediated ubiquitination pathway; nevertheless, these upstream mediators only display mild defects in CSR (Jackson and Durocher, 2013). These results suggest that the critical function of 53BP1 in CSR is mediated independently of its IR-induced accumulation at DNA breaks; however, this interpretation may not be straightforward since RNF8 and RNF168 also mediate the recruitment of BRCA1, which can potentially antagonize and oppose 53BP1.

Current data suggest that the functions of the γ-H2AX/MDC1 and H4K20me/53BP1 interactions during CSR serve at least two similar purposes (Figure 4). As the deficiency of either factor leads to recombination defects between different switch regions without a so-called intra-switch recombination defect between the same switch region, one similar function is to mediate long-range switch recombination of DSBs, which can be nearly 100 kb apart from each other (Bassing et al., 2003; Reina-San-Martin et al., 2007, 2003). Interestingly, while 53BP1 is nearly essential for CSR under normal AID-dependent conditions, only a subtle defect in CSR is observed in 53BP1$^{-/-}$ B cells when DNA breaks at Sμ and Sγ1 are generated with gene-targeted knockin loxp or I-SceI restriction sites near both switch regions in the absence of AID (Bothmer et al., 2010). This result is consistent with 53BP1$^{-/-}$ cells showing very little evidence of spontaneous genomic instability outside of the *Igh* locus (Difilippantonio et al., 2008; Franco et al., 2006; Ward et al., 2004) and supports the notion that 53BP1 function is not an inherent aspect of repairing a DSB but, instead, is important for carrying out the CSR reaction with DNA breaks incurred from AID-induced damage. Thus, for productive CSR, long-range recombination between two switch regions must be favored over intra-switch recombination; however, with the exception of involving H2AX and 53BP1, the mechanistic basis remains largely unanswered.

Another function for the chromatin-associated γ-H2AX/MDC1 and H4K20me/53BP1 interactions is to limit exonuclease-mediated DNA end resection of CSR-associated breaks, which suppresses DNA repair by both homologous recombination and alternative end-joining pathways characterized by increased junctional microhomology and formation of chromosomal translocations (Bothmer et al., 2010; Bunting et al., 2010; Gostissa et al., 2011; Helmink et al., 2011). The suppression of DNA end resection by H2AX or 53BP1 has been shown by comparing the number of resected nucleotides observed in CSR-associated joins from mutant- and control-stimulated B cells at both the endogenous *Igh* locus and at an integrated site-specific DNA break (Bothmer et al., 2010; Bunting et al., 2010). Consistent with these data, G1-arrested developing lymphocytes from H2AX- or 53BP1-deficient mice have been shown to display increased exonucleolytic processing at V(D)J-associated DNA breaks (Difilippantonio et al., 2008; Helmink et al., 2011). Moreover, inhibition of ataxia telangiectasia mutated (ATM) or CtBP-interacting protein (CtIP) can partially rescue the resection and class-switching defects in H2AX- or 53BP1-deficient cells, an activity that appears most apparent in G1 phase cells (Bothmer et al., 2010, 2013; Helmink et al., 2011; Yamane et al., 2013). Even more convincing is the dramatic association of the replication protein A (RPA) ssDNA-binding protein observed by ChIP-seq at *Igh* switch regions undergoing CSR in the absence of H2AX or 53BP1 (Bunting et al., 2012; Yamane et al., 2011, 2013). While robust γH2AX accumulation can be found at the *Igh* locus in normal G1 phase B cells undergoing CSR (Petersen et al., 2001), the majority of RPA is recruited to the *Igh* locus in the S and G2/M phases of the cell cycle (Yamane et al., 2013). Importantly, the extensive DNA end resection observed in H2AX- or 53BP1-deficient B cells is not a general phenomenon with all NHEJ mutants, as KU70 deficiency shows only a subtle increase in RPA association at switch regions (Bunting et al., 2012). Thus, unrepaired CSR-associated DSBs in H2AX$^{-/-}$ or 53BP1$^{-/-}$ B cells persist into S phase, and their increased exonucleolytic processing appears to contribute, at least to some extent, to the CSR defect. As resection progresses, DSBs may then be repaired by A-EJ for a non-productive intra-switch recombination (also called an internal switch deletion) or by HR from an undamaged homologous template to try CSR in the subsequent G1 phase (Figure 4).

Even though both H2AX and 53BP1 deficiencies show evidence of increased DNA end resection, only 53BP1 deletion can rescue the PARP inhibitor sensitivity observed with *Brca1* deficiency (Bothmer et al., 2011). One recent clue as to why deletion of 53BP1, but not H2AX, rescues *Brca1* deficiency and displays a much more severe CSR phenotype comes from the finding that phosphorylation of 53BP1 is critical for CSR and blocking resection (Bothmer et al., 2011; Di Virgilio et al., 2013). Phosphorylated 53BP1 stabilizes RIF1 to DSBs, which appears to help ensure that NHEJ predominates over HR (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Zimmermann et al., 2013). Even so, it largely remains a mystery why 53BP1 is so important for CSR, when its deficiency only shows a subtle defect in the repair of IR-induced DNA breaks. Knowledge gained from a better understanding of 53BP1 and H2AX in CSR will provide additional insight into their general functions as chromatin-binding factors that maintain genome stability.

Recent genetic data suggest that the functions of 53BP1 in promoting NHEJ and suppressing HR are, in fact, separable (Callen et al., 2013). A factor that directly interacts with both γ-H2AX and phosphorylated 53BP1 is the six BRCT domain-containing PTIP (Muñoz and Rouse, 2009; Williams et al., 2010; Yan et al., 2011). One of its tandem BRCT domains (BRCT5/BRCT6) interacts directly with γ-H2AX (Yan et al., 2011), while the folding of both BRCT3/BRCT4 and BRCT5/BRCT6 tandem BRCT domains appear to directly interact with phosphorylated 53BP1 (Gong et al., 2009; Manke et al., 2003; Munoz et al., 2007). IR-induced foci formation of PTIP is dependent on the γ-H2AX/MDC1/RNF8/53BP1 pathway and

is completely abrogated by a single point mutation in BRCT3 of PTIP or by disruption of a subset of ATM/ATR target sites near the N terminus of 53BP1 (Daniel et al., 2010; Gong et al., 2009; Munoz et al., 2007; Callen et al., 2013). However, as the radiosensitivity phenotype of PTIP-deficient cells is mild, its functional role in the DDR remained elusive until recently. Surprisingly, mutation of this subset of ATM/ATR phosphorylation sites near the N terminus of 53BP1 does not impair CSR or RIF1 foci formation but robustly rescues genome instability observed with *Brca1* deficiency (Callen et al., 2013). Indeed, complete loss of PTIP, or, more specifically, loss of the BRCT3 foci-forming function of PTIP, completely restored genome stability in *Brca1* mutant cells (Callen et al., 2013). While direct interaction with γ-H2AX may stabilize PTIP to DNA breaks under conditions still to be determined, PTIP appears to function as a major downstream mediator of phosphorylated 53BP1 to suppress mutagenic DNA repair in S phase (Callen et al., 2013). RIF1, on the other hand, appears to require a different phosphorylation mark further downstream on 53BP1 for its recruitment to sites of DNA damage to mediate productive CSR (Callen et al., 2013). Biochemical elucidation of how PTIP and RIF1 mediate separable functions of 53BP1 to maintain genome stability warrants further investigation.

Widespread AID-Induced Breaks and Tumorigenesis

Uncontrolled AID activity has been shown to cause genomic instability and tumorigenesis with studies employing genetic disruption or overexpression of AID (Muramatsu et al., 2007; Robbiani and Nussenzweig, 2013). Increased AID expression also correlates with higher levels of autoantibodies in several mouse models of lupus and rheumatoid arthritis (Xu et al., 2012). The data support a model in which B cells incurring AID-induced formation of oncogenic chromosomal translocations do not normally survive unless a DNA damage checkpoint pathway that normally eliminates cells with unrepaired DSBs and oncogenic translocations is impaired (Nussenzweig and Nussenzweig, 2010). Consistent with this model, the genomic instability in H2AX$^{-/-}$ mice does not lead to cancer unless a copy of the p53 apoptotic checkpoint tumor suppressor is disrupted (Bassing et al., 2003; Celeste et al., 2003). Similarly, survival of 53BP1$^{-/-}$ mice is greatly compromised upon p53 deficiency (Nussenzweig and Nussenzweig, 2010). Outside of these H2AX and 53BP1 studies, strong evidence for how chromatin structure or regulators thereof may affect the formation of chromosomal translocations is lacking.

Recent work has explored the extent and nature of AID-induced mutations and DSBs at genes outside of the *Igh* locus. Genetic experiments with knockin mice have demonstrated that AID is required for the DSB at *c-myc* that leads to the *c-myc*/*Igh* chromosomal translocation found in Burkitt's lymphoma (Robbiani and Nussenzweig, 2013). In addition to *MYC*, many other genes have been found to be mutated by AID from analysis of genomic DNA sequences from Ung$^{-/-}$Msh2$^{-/-}$ B cells (Liu et al., 2008; Pavri and Nussenzweig, 2011; Robbiani and Nussenzweig, 2013; Yamane et al., 2011). Deep sequencing analyses of AID localization in stimulated B cells have also provided unmatched views of AID-induced DNA damage at non-*Igh* loci (Yamane et al., 2011). It has been proposed that off-target AID sites are repaired by HR (Hasham et al., 2010); however, significant association of RPA and RAD51, as markers of HR-mediated repair, was found only at the *Igh* switch regions undergoing class switching and not at other loci, suggesting that AID recruitment is not sufficient for AID-induced DSB resection (Yamane et al., 2011, 2013). Thus, while AID promotes somatic hypermutation at many genes (Liu et al., 2008; Muramatsu et al., 2007; Robbiani et al., 2009; Yamane et al., 2011), AID-induced DSBs do not appear to occur frequently or to load significant levels of RPA (Petersen et al., 2001; Yamane et al., 2011). Low levels of AID-induced DNA damage at non-*Igh* loci have been proposed to be protected from mutations and genomic instability by high-fidelity, error-free repair mechanisms (Liu et al., 2008; Yamane et al., 2011), and additional investigation is needed to understand how error-prone and error-free DNA repair pathways are targeted to *Igh* and non-*Igh* loci, respectively.

While AID-induced DSBs at non-*Igh* loci do not appear to occur frequently in normal dividing B cells, widespread DSBs caused by AID-induced DNA damage have recently been visualized with certain genetic manipulations. By increasing the mutation load with AID overexpression and increasing DNA end resection with 53BP1 deficiency, AID-induced DNA damage was observed in AIDtg53BP1$^{-/-}$ mice using RPA or RAD51 ChIP-seq at about 150 genes (Hakim et al., 2012; Yamane et al., 2013). Using deep sequencing technologies, approaches to isolate junctions between a chromosomal DSB introduced at a fixed site and other sequences in primary B cells have revealed that DSBs translocate widely across the B cell genome and prefer to be resolved to a break on the same chromosome. Moreover, translocations were preferentially targeted to transcribed regions, most significantly at 2 kb around TSSs (Chiarle et al., 2011; Klein et al., 2011; Zhang et al., 2012), demonstrating that the chromatin environment and/or transcriptional activity are key factors influencing the ability of two DSBs to translocate. Even though AID-dependent SHM at the immunoglobulin variable regions has not previously been detected from B cells stimulated ex vivo (Liu and Schatz, 2009), likely from the result of error-free repair activity (Liu et al., 2008), the sensitivity of translocation sequencing methods has revealed translocation partners fused to the *Igh* variable region, though their dependency on AID was not investigated (Klein et al., 2011). These sequencing experiments also revealed the surprising identification of translocations, albeit at low levels, at Sμ and Sγ1 in AID$^{-/-}$ B cells, indicating that DSBs at *Igh* can occur at a low frequency in the absence of AID (Chiarle et al., 2011). Among the translocation hotspots that partner with the *Igh* locus, the number of translocations per hotspot was directly proportional to the amount of chromatin-associated RPA or RAD51 at these regions (Hakim et al., 2012; Yamane et al., 2013). These results suggest not only that *Igh* translocates to many sites in the genome that incur DSBs, but also that, in addition to proximity, frequent DSBs drive recurrent translocations. All together, the data provide indisputable evidence that AID-induced mutation can lead to widespread DSBs and formation of chromosomal translocations and begin to address how the chromatin environment impacts aberrant resolution of DNA breaks.

Other Implications for Chromatin in AID-Independent Replication Stress

CSR-associated DNA breaks are normally resolved in the G1 phase of the cell cycle but can persist into the S and G2/M phases and be visualized cytogenetically in metaphase spreads upon disruption of one of a number of DDR or C-NHEJ factors (Boboila et al., 2012; Nussenzweig and Nussenzweig, 2010). One group of investigators recently visualized the persistence of CSR-associated breaks in each cell-cycle phase by employing ChIP-seq of γ-H2AX and RPA from stimulated B cells sorted based on their DNA content (Yamane et al., 2013). The persistence of AID-mediated DNA breaks into S phase allows the possibility of these breaks to aberrantly join with DSBs generated as a result of replication stress. Indeed, many recurrent mutations in B cell lymphoma are not associated with AID activity (Robbiani and Nussenzweig, 2013; Rui et al., 2011); however, genes that are large and highly transcribed are thought to cause problems for the replication machinery and be a source of DSBs that must be properly cared for to suppress tumorigenesis (Helmrich et al., 2011; Prado and Aguilera, 2005). Using an unbiased approach to discover sites of recurrent DNA lesions during early replication through analyses of γ-H2AX, RPA, BRCA1, and SMC5 ChIP-seq localization data, a new class of early replication fragile sites (ERFSs) was recently identified in stimulated B cells (Barlow et al., 2013). ERFSs break spontaneously at a low frequency but are acutely induced upon treatment with hydroxyurea or ataxia telangiectasia- and Rad3-related (ATR) inhibition or in response to oncogenic stress (Barlow et al., 2013). While common fragile sites are characterized by their late replicating and being within a condensed chromatin structure marked by histone hypoacetylation (Ozeri-Galai et al., 2012), ERFSs replicate early, show hallmarks of open chromatin structure, and are gene rich (Barlow et al., 2013). Although ERFS breakage is AID independent, some of the regions that are sensitive to replication stress encompass hotspots for AID-induced damage. Cytogenetic examination of metaphase spreads from stimulated AIDtg53BP1$^{-/-}$ B cells, which allow *Igh* breaks generated in G1 to persist into S phase, led to the identification of a chromosomal translocation between *Igh* and an ERFS in primary cells (Barlow et al., 2013). Importantly, greater than 50% of common amplifications and deletions observed in human diffuse large B cell lymphoma map to ERFSs (Barlow et al., 2013), suggesting that ERFSs may be a significant source of genomic instability that act together with AID-induced DNA damage to promote lymphomagenesis.

One ERFS identified using this approach was Bcl-2 (Barlow et al., 2013), an apoptotic regulator located within the so-called major breakpoint region (mbr) that contains clusters of CpG dinucleotides, adopts a single-stranded non-B DNA structure, and is translocated to *Igh* in 70%–95% of follicular lymphomas and 20%–30% of diffuse large B cell lymphomas (Raghavan et al., 2004; Tomita, 2011). The observation that CpG dinucleotides can be found within 40%–70% of breakpoints at chromosomal translocations in immature human B cell lymphomas recently led to a proposal that DSBs at Bcl-2 and other genes may arise from sequential action of AID and the structure-specific nicking activity of the RAG1/RAG2 complex during early B lymphocyte development (Tsai et al., 2008). Similar to Bcl-2, other ERFSs are also enriched for CpG dinucleotides (Barlow et al., 2013). As such, it is possible that replication stress further stabilizes the single-stranded conformation of the Bcl-2 mbr (Tsai et al., 2008), which may subsequently be more prone to slippage and collapse during DNA replication.

Thus, AID-induced DNA damage and replication stress can both lead to widespread DSBs that, if not repaired by the NHEJ or HR pathways, can become partners for chromosomal translocations. While chromosomal translocations occur regularly in primary dividing cells, only upon deregulation of cell growth or survival will the translocation lead to tumorigenesis. It is clear that highly transcribed genes displaying H3K4me3, H3ac, and H3K36me3 are prone to breakage and forming translocations (Barlow et al., 2013; Klein et al., 2011), but whether or not the histone marks directly aid in DSB formation or aberrant repair remains unclear. Thus, future work will determine the mechanisms by which chromatin regulates the repair of AID-induced and replication-induced DNA damage.

Concluding Remarks

Chromatin biology touches on all facets of developmental and disease biology, and the DNA repair field is no exception. The ever-increasing number of proteins implicated in the DNA damage response continues to call for detailed genetic studies to clearly determine which proteins and PTMs are physiologically relevant and which may potentially be targeted for therapeutics. With respect to accessibility for AID, whether a specific chromatin-mediated activity is required for this DNA rearrangement event remains to be clarified. Moreover, whether or not germline transcripts are the cause or the effect of chromatin changes at antigen receptor loci remains unclear. With respect to recombination and repair of *Igh*-associated DNA breaks, it is clear that histone H2AX and methyllysine recognition by 53BP1 play critical roles within the context of chromatin; however, we have only scratched the surface, with much still to be learned. For example, we understand next to nothing about the roles of many of the other myriad histone PTMs (Tan et al., 2011). Since multiple genes encode most histones, generating useful mammalian models with a single amino acid point mutation in a histone has not been feasible. Instead, we now have the technological means to explore this exciting avenue of research with detailed mechanistic studies in physiologically relevant model systems using deep sequencing and proteomics, which promise to reveal new insight. Along the way, we will also find out whether AID has additional functions beyond antibody diversification.

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GENOMIC INSTABILITY IN CANCER

End-joining, translocations and cancer

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Abstract | Fusion genes that are caused by chromosome translocations have been recognized for several decades as drivers of deregulated cell growth in certain types of cancer. In recent years, oncogenic fusion genes have been found in many haematological and solid tumours, demonstrating that translocations are a common cause of malignancy. Sequencing approaches have now confirmed that numerous, non-clonal translocations are a typical feature of cancer cells. These chromosome rearrangements are often highly complex and contain DNA sequence from multiple genomic sites. The factors and pathways that promote translocations are becoming clearer, with non-homologous end-joining implicated as a key source of genomic rearrangements.

Non-homologous end-joining
(NHEJ). Joining of DNA double-strand breaks without extensive sequence homology by ligation of DNA ends.

Chromothrypsis
A highly complex chromosome rearrangement event characterized by extensive re-assortment of genetic fragments from one or more chromosomes.

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The root cause of the mutations that lead to cancer is genomic instability[1]. Therefore, a great deal of research has focused on cellular processes that can contribute to or that can counteract genomic instability. Although the importance of genetic changes in driving cancer has been appreciated for almost 100 years, recent technological advances have substantially increased our ability to study cancer-associated mutations. Furthermore, by studying DNA repair pathways that normally suppress the genomic instability that leads to mutation, we now better understand why mutations arise, and in several cases how we can manipulate cells to reduce the rate of mutation.

In this Review we focus on how translocations arise, with a particular emphasis on how non-homologous end-joining (NHEJ) causes the appearance of many chromosome rearrangements, including the spectacularly complex chromosome translocations that are associated with chromothrypsis[2].

The nature of translocations

A translocation is an abnormal chromosome region that contains rearranged genetic material, usually from two non-homologous chromosomes (BOX 1; FIG. 1). Translocations are not exclusive to cancer cells; screening of cells from developing embryos has revealed that substantial numbers of embryos (in the order of 0.7 per 1,000 live births) have cells that contain translocations[3,4]. Although in some cases these *de novo* translocations are associated with developmental abnormalities, many balanced translocations do not cause noticeable pathology, suggesting that balanced translocations are well tolerated in many instances. Untransformed primary mouse blood cells also contain a wide range of chromosomal translocations[5–8]. However, translocations are particularly common in cancer cells. Many translocations have been catalogued[9,10] and are listed in databases such as the Database of Chromosome Rearrangements in Disease (dbCRID) and the Catalogue of Somatic Mutations in Cancer (COSMIC). Some translocations fuse genetic elements from different genomic locations to form pathological gene fusions that deregulate cell growth, such as the breakpoint cluster region–Abelson tyrosine-protein kinase 1 (*BCR–ABL1*) translocation in chronic myeloid leukaemia. Historically, most of these gene fusions were found in haematological malignancies; however, a growing number of such mutations have been found in solid tumours[9]. This is exemplified by prostate cancer, in which at least 40% of cases feature translocations between transmembrane protease, serine 2 (*TMPRSS2*) and a gene encoding the ETS transcription factor, *ERG*[11].

The frequency of recurrent gene fusions varies depending on the specific type of cancer, but currently known translocations are estimated to drive ~20% of cancer cases[9]. Next-generation sequencing of genomes and transcriptomes from primary human cancer cells is revealing new gene fusions that may be involved in driving tumorigenesis, including new examples found in colorectal carcinoma, breast cancer and acute lymphoblastic leukaemia (ALL)[12–14]. Nonetheless, sequencing has shown that somatic mutations affecting the sequence of genes are considerably more common than chromosome rearrangements[12–17].

Sequencing efforts have also revealed that cancer genomes do not typically contain a discrete number of coherent reciprocal translocations. Tumour cells more commonly contain a large number of complex translocations, featuring interchromosomal and intrachromosomal

Key points

- Translocations that create neomorphic fusion genes occur in both lymphoid malignancies and solid tumours.
- A large number of translocations do not encode fusion genes and may not contribute to malignancy.
- Translocations frequently contain complex, clustered sequence rearrangements, similar to chromothrypsis, and may also contain genetic material from several different chromosomes.
- Many translocations arise as a consequence of 'classical' or 'alternative' pathways of non-homologous end-joining.
- Mammalian cells have regulatory systems to bias DNA repair towards repair pathways that are less likely to contribute to translocation.
- Frequency of DNA breakage is the metric that best predicts the likelihood of a particular genomic site being involved in a translocation.
- Therapeutic intervention to reduce translocation frequency is a potential mechanism for reducing the risk of cancer.

rearrangements (FIG. 1). In most cases, it is not possible to draw definite conclusions about the mechanism or extent by which any one of these individual translocations contributes to the malignancy of the cancer cell. Furthermore, the frequency and the type of translocations are not always shared among tumours of the same class. Sequence data from primary breast cancers showed between zero and 29 translocations per case[13]. Squamous cell lung cancer cells have a higher rate of translocations, with an average of 165 somatic rearrangements per cell, compared with 98 rearrangements per cell in non-small cell lung carcinoma and 90 rearrangements per genome in prostate tumours[15–17]. Rearrangements in cancer cells affect genic and non-genic DNA at approximately equal rates; however, a study in prostate cancer cells found an enrichment of rearrangements in transcribed regions, as measured by RNA polymerase II chromatin immunoprecipitation (ChIP)[15]. A study in breast cancer cells also reported an increased rate of rearrangements within the total area (including introns) of protein-coding genes[13]. A minority of translocations form gene fusion events, but translocations may also contribute to tumorigenesis by interrupting the sequence of tumour suppressor genes, as observed for the tumour suppressor tetratricopeptide repeat domain 28 (*TTC28*) in certain cases of colorectal carcinoma[12]. As is the case with mutations affecting gene sequence, many translocations seen in cancer cells are probably bystander mutations as opposed to drivers of the disease. The number of translocations in tumour cells from a cohort of patients with lung adenocarcinoma was not found to correlate with clinical outcome[17]. Hence, tumours with a small number of translocations can be more aggressive and more difficult to treat than tumours with many translocations. Other types of mutations in addition to translocations clearly play an important part in driving the growth and survival of cancer cells.

The frequency and complexity of cancer-associated translocations has required the development of new bioinformatic tools to analyse and display the huge volume of data that is being generated by cancer genome-sequencing projects[18] (FIG. 1). An example of a spectacular genomic rearrangement was revealed in a case of chronic myeloid leukaemia (CML) that had 42 intrachromosomal rearrangements affecting chromosome 4q[19]. Such highly complex, clustered translocations are referred to as chromothrypsis. These rearrangements can affect one or more chromosomes in a cell and are thought to be generated in a single catastrophic event. An initial estimate indicates that as many as 3% of all cancers exhibit such clustered rearrangements[20]. It is plausible that the same mechanisms that cause chromothrypsis also cause the complex translocations seen in other cancer cells[2]. Complex translocations similar to chromothrypsis have been described as a result of sequencing the genomes of cells from prostate cancer and ALL[14,21].

NHEJ as a source of genomic instability

Several pathways have been proposed to be involved in the formation of translocations[22] (FIG. 2). These pathways include NHEJ, breakage–fusion–bridge cycles (BOX 2; FIG. 3) and replication-based mechanisms, such as break-induced replication (BIR)[23]. Replication-based mechanisms are proposed to cause translocations by switching the extending DNA strand from its template sequence to

Box 1 | **Types of chromosomal translocations**

The development of techniques for visualizing and staining chromosomes using dyes such as quinacrine and Giemsa led to the first identification of translocations in the 1950s, and important disease-causing rearrangements are still being discovered today. A chromosome that contains a translocation is termed a 'derivative chromosome', and the nature of the rearrangements that affect that chromosome are described by a systematic nomenclature. Robertsonian translocations are those in which the long arms of two acrocentric chromosomes are joined around a single centromeric region. Reciprocal translocations describe the exchange of genetic material between two chromosome arms. Such translocations can be classified as 'balanced' or 'unbalanced' depending on whether the translocation affects the copy number of any section of the genome, with a balanced translocation causing no change to overall copy number.

Many well-known pathological translocations fall into the class of apparently balanced, reciprocal translocations between two non-homologous chromosomes. This group includes the Philadelphia chromosome, t(9;22) (translocation between chromosome 9 and 22) found in chronic myeloid leukaemia; a translocation between chromosomes 11 and 22, t(11;22), which is seen in 85% of cases of Ewing's sarcoma; and a translocation of chromosomes 8 and 14, t(8;14), which is seen in 85% of cases of Burkitt's lymphoma. Translocations such as these promote cancer by deregulating the expression of key cellular transcription factors and signalling modulators to cause uncontrolled growth. In addition to the recurrent t(9;22) translocation in chronic myeloid leukaemia, which causes overexpression of ABL1 kinase, the t(11;22) translocation in Ewing's sarcoma causes deregulated activity of Friend leukaemia integration 1 (FLI1), an ETS transcription factor, whereas t(8;14) in Burkitt's lymphoma is a translocation that causes the overexpression of the mitogenic MYC transcription factor.

Break-induced replication (BIR). A modified homology-based repair pathway in which a broken DNA end is repaired by copying a large amount of sequence from an undamaged homologous partner, potentially leading to copying of the entire homologous sequence from the site of damage to the end of the chromosome.

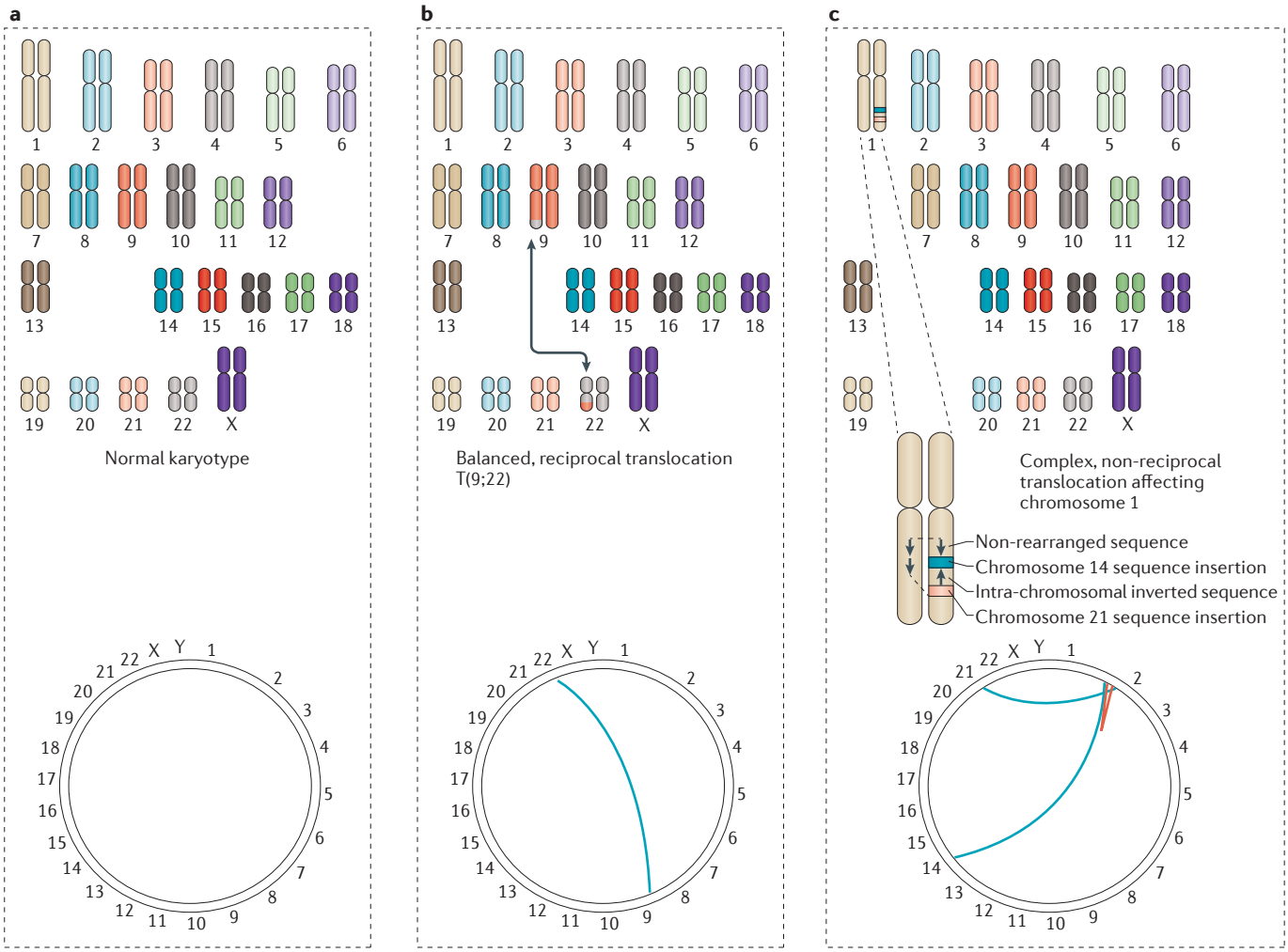

Figure 1 | **Visualizing translocations.** **a** | The normal human chromosome set contains no rearrangements between chromosomes. The Circos plot[18] shows this as a ring with the uninterrupted sequence of the chromosome running around the circumference. **b** | Certain cancer cells contain balanced, reciprocal translocations, which join sequence from different chromosomes, such as the t(9;22) translocation from chronic lymphocytic leukaemia, which exchanges sequence from chromosomes 9 and 22. Viewed as a Circos plot, this translocation can be visualized as a line connecting the breakpoints of the translocation on chromosomes 9 and 22. **c** | Many translocations are more complex rearrangements involving multiple chromosomes. In this example, chromosome 1 contains a rearrangement involving translocated sequences from chromosomes 14 and 21 and an internal sequence inversion. Such complex translocations can be pictured using the Circos plot, in which the blue lines indicate interchromosomal translocations and the red line shows the intrachromosomal inversion.

another homologous template during DNA replication, potentially resulting in a non-homologous sequence being copied into the new DNA strand[24,25]. However, in the absence of an appropriate inducible model or genetic evidence for the requirement of specific factors in mediating replication-based translocations, it is challenging to quantify the contribution of such pathways to the overall frequency of translocations. Mechanisms based on homologous recombination may also cause translocations, such as non-allelic homologous recombination (NAHR), which has been implicated in chromosome rearrangements that occur in the germ line[26].

Translocations, in particular those translocations that generate gene fusions, are often assumed to form because of the joining of DNA double-strand breaks that arise at different sites on non-homologous chromosomes. In this case, the double-strand breaks are joined by an endogenous DNA repair pathway such as NHEJ (FIG. 4). In mammalian cells, the best-characterized pathway for NHEJ, which has become known as 'classical' NHEJ (C-NHEJ), initiates through the binding of a heterodimer comprised of Ku70 (also known as XRCC6) and Ku80 (also known as XRCC5) to broken DNA ends[27]. A complex of DNA-dependent protein kinase catalytic subunit (DNA-PKcs; also known as PRKDC) and Artemis (also known as DCLRE1C) subsequently binds to the Ku70–Ku80–DNA complex and processes the DNA end through the nuclease activity of Artemis. Finally, a complex comprised of XRCC4-like factor (XLF; also known as NHEJ1), XRCC4 and DNA ligase 4 (LIG4)

Non-allelic homologous recombination
(NAHR). Recombination between repetitive regions at different genomic sites that leads to chromosome rearrangements, as seen in genetic diseases such as Charcot–Marie–Tooth syndrome.

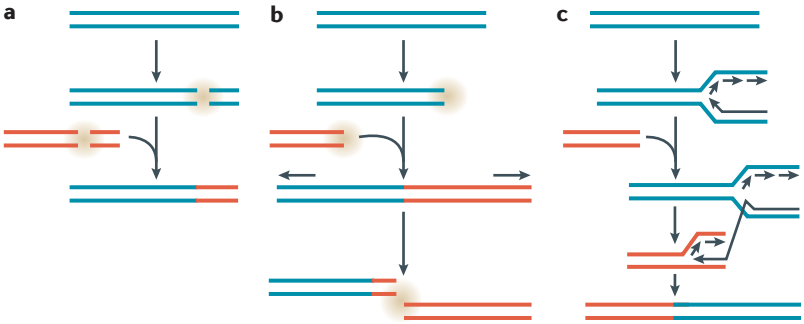

Figure 2 | **Pathways to translocation.** **a** | Balanced reciprocal translocations are hypothesized to form as a consequence of the fusion of two double-strand breaks that arise in the same cell. Following the appearance of DNA double-strand breaks, a signalling pathway is activated, which leads to the ligation of the free DNA ends that is mediated by factors of the non-homologous end-joining pathway. Red and blue strands represent different chromosomes, which may become incorrectly joined by endogenous repair pathways. **b** | Telomere uncapping or attrition generates a DNA double-strand break response, which potentially leads to the fusion of telomeres, generating end-to-end fusions. During anaphase, dicentric fusion chromosomes are pulled apart, leading to the formation of translocations and double-strand breaks. Broken chromosomes act as substrates for additional rounds of fusion and breakage, generating increasingly complex translocations. **c** | Hypothetically, translocations could arise through a replication-based mechanism by 'switching' of the DNA replication machinery to a site on a different chromosome with some degree of sequence homology to the original template. Extension of the replication fork (short arrows) at a site on a different chromosome (crooked arrow) would lead to a composite daughter strand being produced, which would contain sequence from two chromosomes. This composite chromosome would appear as a translocation. Highly complex translocations could be generated by multiple template switching events, generating an aberrant chromosome that contains sequence from several different parts of the genome.

joins the DNA ends (FIG. 4). The importance of the NHEJ pathway in maintaining genomic stability is known from genetic studies in mice[28–30] and from individuals with mutations in key NHEJ genes (TABLE 1).

Despite the appearance of chromosomal translocations in cells that lack NHEJ activity, several lines of evidence suggest that, in certain cases, NHEJ contributes to the appearance of chromosomal translocations. Deficiency in RAD18 makes cells hypersensitive to camptothecin, an agent that is used in certain chemotherapy regimens on the basis of its ability to cause DNA double-strand breaks. However, this hypersensitivity is relieved by suppressing NHEJ[31]. Mutations in *BRCA1*, which is required for homologous recombination, or in any of the Fanconi anaemia complementation group (FANC) genes, which excise DNA interstrand crosslinks, predispose affected individuals to cancer owing to the requirement of these factors for normal DNA repair. However, several reports have concluded that ablation of NHEJ factors such as Ku70, Ku80 or LIG4 reduces genomic instability and the appearance of chromosome rearrangements in BRCA1- or FANC-deficient cells[32–35]. Chemical inhibition of DNA-PKcs has also been reported to reduce genomic instability in cell lines lacking BRCA1 and BRCA2 (REF. 36). Collectively, these results suggest that, when mammalian DNA repair pathways are defective, the NHEJ pathway can increase the amount of genomic instability and, therefore, accelerate the accumulation of mutations that contribute to cancer.

Genomic sequencing indicates that up to 50% of ovarian carcinoma cells have mutations that affect the homologous recombination pathway, making these cells particularly vulnerable to aberrant DNA repair by NHEJ[37]. Cells from patients with breast cancer have a higher than expected frequency of mutations in *FANCC*, Bloom's syndrome, RecQ helicase-like (*BLM*; also known as *RECQL3*) and *XRCC2* (REFS 38–40). These genes are essential for error-free repair of DNA damage, hence cells with these mutations may over-use NHEJ for repair, leading to further accumulation of genetic abnormalities. Another form of evidence that NHEJ is important for chromosome translocations has come from the study of the genetic requirements for the fusion of uncapped telomeres (BOX 2). NHEJ is also considered to be the mechanism that underlies the complex pattern of translocations and rearrangements seen in chromothrypsis[19]. Therefore, NHEJ has an important role in shaping the genome of the cancer cell by contributing to error-prone pathways of DNA repair that lead to the appearance of mutation.

Classical versus alternative end-joining pathways

Early studies on the characteristics of end-joining activities in mammalian cells demonstrated the presence of two classes of products: those formed from the simple ligation of DNA ends and those in which small sections of shared sequence identity (microhomology) at the joined ends could be observed[41,42] (FIG. 4). Yeast studies support the importance of Ku70–Ku80 for NHEJ but additionally show that in the absence of these factors an alternative activity can mediate joining using microhomology, although with some deletion of DNA sequence around the break site[43–45]. Subsequent biochemical data and assays with end-joining substrates with different amounts of terminal homology showed that the joining of ends with 6–8 bp of homology is independent of the Ku-mediated C-NHEJ[46,47] and instead requires a NHEJ pathway called alternative end-joining (A-EJ) or microhomology-mediated end-joining (MMEJ). The existence of A-EJ accounts for translocations and chromosome rearrangements in cells that lack Ku70, Ku80 or LIG4 (REF. 29). Notably, mice with targeted knockouts of Ku70–Ku80, XRCC4 or LIG4 in combination with p53 deficiency develop tumours with translocations featuring microhomology[48,49]. Microhomology was also reported in 85% of the translocations that were induced using a translocation reporter system in mouse embryonic stem cells[50]. Therefore, A-EJ seems to be capable of producing translocations, particularly when C-NHEJ is deficient. In a system that measured the frequency of translocations between the immunoglobulin heavy chain complex (*Igh*) and *Myc* in mouse B cells, deletion of Ku70 or LIG4 actually increased the rate of translocation, with A-EJ apparently providing the joining activity[51]. Translocations were also increased in a reporter system in mouse embryonic stem cells when XRCC4–XLF was inactivated[52]. These results suggest that NHEJ causes a low rate of translocations, but in its absence A-EJ becomes active and produces an increased number of chromosome rearrangements.

A-EJ is of particular interest because microhomology signatures have been reported at the breakpoints of chromosome rearrangements in primary human cancer cells[53,54]. This raises the possibility that A-EJ, or some other microhomology-based mechanism, is responsible for the formation of translocations. The amount of microhomology used in repair of a DNA double-strand break is the standard measure for distinguishing between C-NHEJ and A-EJ, but it is unclear how much microhomology is optimal for each pathway. Understanding the importance of A-EJ in the formation of translocations will require better characterization of the components of the pathway. The identification of factors that are required for A-EJ in mammalian cells has been aided by studies in yeast, which have suggested that factors such as meiotic recombination 11 (Mre11), Rad50 and Sae2 are involved in A-EJ[53]. Studies using translocation reporter constructs in mouse embryonic stem cells have shown that the frequency of translocations between induced double-strand breaks on different chromosomes is reduced after knockdown of CtBP-interacting protein (CtIP; also known as RBBP8), an exonuclease that is considered to be the closest mammalian homologue of Sae2 (REF. 55). Furthermore, the translocations that do occur show a reduced amount of microhomology at the breakpoints, supporting a role for CtIP in a pathway that produces translocations using microhomology.

A role for CtIP in A-EJ is plausible according to a model in which limited exonuclease resection of DNA double-strand breaks is necessary to uncover stretches of microhomology that can anneal and mediate joining (FIG. 4). Intrachromosomal joining assays in mammalian embryonic stem cells and cell lines have likewise supported an involvement of Mre11 in Ku-independent end-joining using microhomology[56,57]. However, although modulation of end resection seems to be a key regulator of A-EJ, data from mouse B cells measuring induced intrachromosomal rearrangements showed no reduction of microhomology-mediated joining in cells after CtIP knockdown or MRE11 inhibition[58]. Therefore, the essential genetic make-up of A-EJ is an ongoing question in the field, and multiple redundant processes might contribute to A-EJ. The DNA end-binding factor Ku has recently been suggested to have a key regulatory role in suppressing the use of A-EJ, as depletion of human Ku86 was found to increase the use of A-EJ in cells lacking other C-NHEJ factors[59].

As A-EJ is active in the absence of LIG4, much interest has focused on which of the other two mammalian ligase enzymes (LIG1 or LIG3) is required for the joining of DNA ends in A-EJ. Depletion of either LIG1 or LIG3 reduces the use of microhomology-mediated end-joining of cut plasmids in cell-free extracts[60]. Cells with a specific deficiency in nuclear LIG3 show a reduced frequency of translocations between targeted double-strand breaks on chromosomes 6 and 11, with the small number of remaining translocations showing a reduced use of microhomology[61]. This supports a role of LIG3 for mediating A-EJ translocations in mammalian cells and suggests that A-EJ is active even when all of the factors of the C-NHEJ pathway are present. This study further demonstrated that LIG1 can act as a back-up ligase for LIG3 in A-EJ because depletion of both LIG3 and LIG1 together reduces translocations to a lower rate than that seen in nuclear LIG3-deficient cells. However, studies of the conditional deletion of XRCC1, the cofactor of LIG3, in B lymphocytes have produced conflicting data regarding the importance of LIG3 in A-EJ[62]. In B cells with deficiencies in C-NHEJ, deletion of XRCC1 or knockdown of LIG3 had no effect on translocations between *MYC* and *IGH*. The relative importance of LIG3 and LIG1 in A-EJ is thus still somewhat unclear.

Although A-EJ is an important pathway for the formation of translocations, several lines of evidence suggest that C-NHEJ still accounts for most rearrangements. First, the measured amount of microhomology found in translocation reporter cell lines is quite low, with a mean of 1.36 bp[50]. Second, in two different inducible systems that generate experimental interchromosomal translocations, microhomology-mediated joining was observed in a minority of cases[63,64]. Third, data from next-generation sequencing projects involving human cancer patients indicate a minor role for A-EJ. For example, one recent study used next-generation sequencing technology to characterize the breakpoints of 52 germline chromosomal rearrangements from human patients[65]. Most of these rearrangements were thought to be balanced translocations. However, at the molecular level they almost invariably featured the deletion of genetic sequence at the breakpoint junction. A significant number of the translocations were not formed by the simple joining of DNA breaks but involved local fragmenting of the DNA with the reassembly of inverted local sequence at the final translocation join. Of the 141 breakpoints

Box 2 | **Telomeres and translocations**

Telomeres normally protect the end of chromosomes, but incipient tumour cells are known to have acutely short telomeres[120,121]. When telomeres become shortened or uncapped by loss of shelterin, the chromosome end is signalled as a double-strand break[122]. Normally, p53 signalling triggers apoptosis in response to this signal, but in telomerase-null mice in the absence of p53, end-to-end chromosome fusions are observed that correlate with a high frequency of epithelial cancer[123]. End-to-end chromosome fusions cause genomic instability because the different centromeres of the fused chromosome are pulled in opposite directions during cell division eventually causing the fused chromosome to break, generating translocations and new DNA ends that form substrates for additional breakage–fusion–bridge cycles[124] (FIG. 3). Key intermediates in this process — that is, dicentric chromosomes and anaphase bridges — have been observed in primary human tumours[125]. Complex translocations in B cells with combined genetic deletion of DNA repair genes and p53 also seem to be derived from cycles of breakage–fusion–bridge[48,49]. These complex translocations feature amplification of the *MYC* oncogene, which is an essential driver of tumorigenesis in these cells. End-to-end fusion of uncapped telomeres is also dependent on the non-homologous end-joining factors Ku70 and DNA ligase 4 (REFS 126,127). Whereas loss of the shelterin component telomeric repeat-binding factor 2 (TRF2) causes chromosome fusion by classical non-homologous end-joining DNA repair pathways[128], alternative end-joining-mediated chromosome fusions are observed in TRF2-deficient cells when Ku80 is absent. In mice, the shelterin proteins TRF1, tripeptidyl-peptidase 1 (TPP1), protection of telomeres protein 1A (POT1A) and POT1B combine with TRF2 to suppress alternative end-joining events[78,129]. Although the importance of this effect in human cancer is not fully clear, mouse models of cancer that arises from defective telomere function share many common genomic features with human tumours[130]. Hence, both classical and alternative end-joining pathways are active in causing chromosome rearrangements that arise from end-to-end fusions.

identified through next-generation sequencing, just 30.5% had regions of microhomology. In addition, chromosome rearrangements in prostate cancer cells do not usually contain microhomology sequences, and in breast cancer cells microhomology is generally either absent or limited to 2 bp or less[13,21]. These findings suggest that microhomology-based mechanisms are responsible for a minority of *de novo* human translocations.

Choice of homologous recombination or NHEJ

C-NHEJ is the only double-strand break repair pathway that can join DNA ends with no homology at the repair site. Furthermore, C-NHEJ acts at blunt or minimally processed DNA ends, whereas some degree of resection of the double-strand break is required for homologous recombination, single-strand annealing (SSA) and A-EJ. The regulation of double-strand break resection therefore acts as the key determinant in committing the repair of a double-strand break to C-NHEJ or to a homology-based pathway[66,67]. One potential method for the regulation of resection is kinetic: resection only proceeds after initial attempts at NHEJ of double-strand breaks have failed. This hypothesis has been supported by multiple lines of evidence using immunofluorescence to detect the accumulation of repair factors at break sites, plasmid rejoining assays and reporter constructs[68–70]. NHEJ seals DNA breaks with minor nucleotide deletions and additions at the breakpoint and is capable of joining DNA breaks on different chromosomes. It is thus surprising that cells use 'quick and dirty' repair by NHEJ rather than the slower, more accurate repair by homologous recombination. Homologous recombination, which is template-based and much less error-prone than NHEJ, might be expected to be the preferred pathway for the faithful repair of double-strand breaks.

Resection of a DNA double-strand break is initiated by MRE11 as part of a complex with RAD50 and Nijmegen breakage syndrome protein 1 homologue (NBS1) in mammals (the MRN complex) or by Mre11 as part of a complex with Rad50 and Xrs2 in yeast cells (the MRX complex). Resection becomes extensive following the action of CtIP. Exonuclease 1 (EXO1) and BLM–DNA2 have also been reported to generate single-stranded DNA overhangs at break sites in mammalian cells. Cyclin-dependent kinase (CDK) signalling regulates the activity of the resection apparatus, such that it is mainly active in the S and G2 phases of the cell cycle. Nonetheless, components of the NHEJ pathway remain active in S phase and G2 phase cells and compete with homologous recombination for the repair of double-strand breaks[69,71]. Extensive resection can also occur in G1, at least in the absence of p53-binding protein 1 (53BP1) and H2AX[72,73]. Thus, in addition to CDK activity, other levels of regulation must be present to ensure the use of error-free homologous recombination versus mutagenic NHEJ.

The DNA damage response factor 53BP1 is a key regulator of DNA end resection in mammalian cells. *53bp1*$^{-/-}$ cells are mildly sensitive to ionizing radiation compared with other cells with deficiencies in the NHEJ pathway, but *53bp1*$^{-/-}$ B cells have a marked defect in their ability to mediate class switch recombination (CSR)[74,75]. Several lines of evidence suggest that 53BP1 may act to repress homologous recombination through blocking resection[33,34,58,73,76–78]. It is not clear whether 53BP1-mediated blocking of resection achieves the rapid repair of breaks at the expense of potential mutagenicity or whether it has evolved to enable the repair of induced double-strand breaks during the assembly of antigen receptor genes. 53BP1 inhibits the resection of DNA double-strand breaks by recruiting two proteins: Pax transactivation domain-interacting protein (PTIP) and RAP1-interacting factor 1 (RIF1)[79–83,131]. PTIP and RIF1 bind to sites in 53BP1 that are phosphorylated by the damage-response kinase ataxia telangiectasia mutated (ATM). 53BP1–RIF1 represses the recruitment of BRCA1 to DNA damage sites in the G1 phase of the cell cycle,

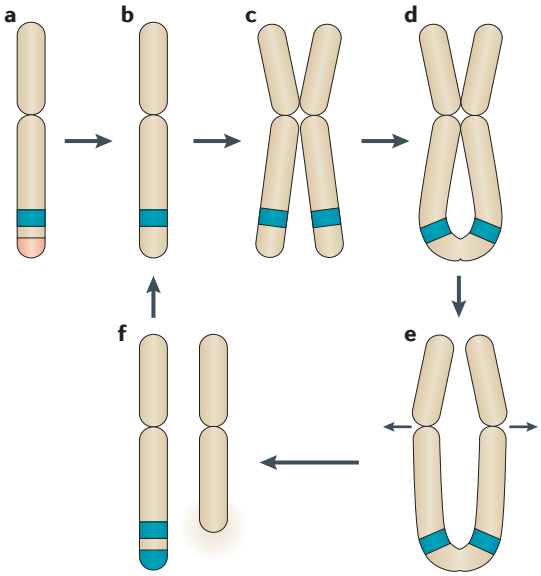

Figure 3 | **Oncogene amplification by breakage–fusion–bridge cycles.** **a** | Chromosomes are normally protected by telomeres (indicated by the blue box). A subtelomeric oncogene (shown in red) can become amplified by breakage–fusion–bridge cycles. **b** | Telomere loss or double-strand breakage creates an unprotected DNA end, which triggers a DNA damage response. **c** | Cancer cells with checkpoint defects will continue to grow despite DNA damage signalling, leading to the duplication of the broken chromosome. **d** | Ligation of broken chromatid ends produces an 'anaphase bridge', with a chromatin connection between the two sister chromatids. **e** | As chromatids are drawn apart during anaphase, the anaphase bridge is subjected to increasing stress as centromeres are pulled to opposite poles of the dividing nucleus. **f** | Eventually, the anaphase bridge will shear, producing uneven derivative chromosomes. One derivative chromosome may capture sequence, including a second copy of the oncogene from the broken sister chromatid. The broken chromosomes can act as substrates for further breakage–fusion–bridge cycles (parts **b**–**f**), potentially leading to the dramatic amplification of oncogenes near telomeric sites. Oncogene amplification is a driver of malignant cell growth. If breakage–fusion–bridge cycles are combined with the fusion of double-strand breaks from other chromosomes, complex translocations can be built up that feature sequence from multiple chromosomes.

Class switch recombination (CSR). A region-specific deletional recombination reaction that replaces one switch region with another. This allows the production of different immunoglobulin isotypes.

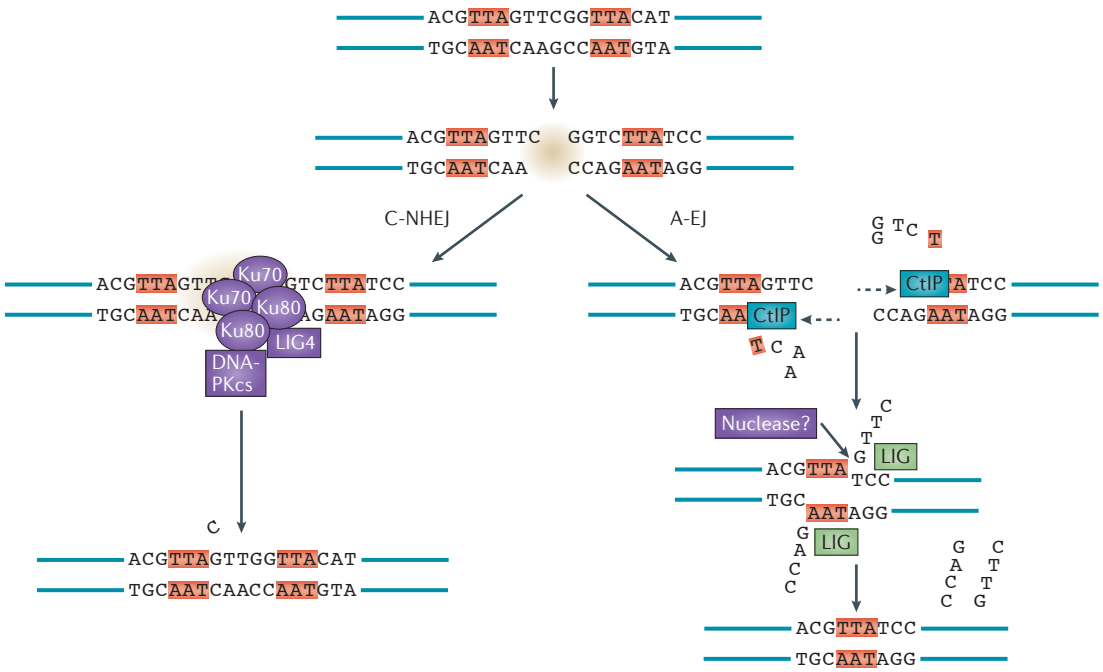

Figure 4 | **Steps in classical and alternative end-joining.** On the appearance of a DNA double-strand break, two pathways can be active. Classical non-homologous end-joining (C-NHEJ) involves the binding of Ku70–Ku80 to the DNA break, followed by the recruitment of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and several other factors that mediate blunt-end ligation of the break by DNA ligase 4 (LIG4). This process has no sequence requirements and may cause small-scale mutation, such as the addition or the deletion of a small number of nucleotides at the break junction. Alternative end-joining (A-EJ) involves exonucleolytic processing of the double-strand break to reveal stretches of potentially complementary sequence (microhomology; indicated in red) on either side of the break. This resection process may be mediated by the exonuclease CtBP-interacting protein (CtIP). Following base pairing at regions of microhomology, the ends are joined by an undetermined ligase enzyme (LIG).

whereas BRCA1, in coordination with CtIP, prevents the accumulation of 53BP, RIF1 and PTIP at break sites during S phase and G2 phase (FIG. 5). In the absence of RIF1, there is a marked defect in CSR, whereas loss of PTIP rescues genome stability in BRCA1-deficient cells. Both RIF1 and PTIP promote end-end fusions of unprotected telomeres. These findings demonstrate the complex regulation of double-strand break repair pathway choice in mammalian cells and reinforce the idea that proper choice is essential for maintaining genome integrity.

DNA-PKcs, which associates with C-NHEJ factors in mammalian cells but which is not present in yeast, is a candidate regulator of NHEJ in mammalian cells. As measured by reporter substrates, increased expression of DNA-PKcs represses homologous recombination, but this effect is not seen with mutant forms of DNA-PKcs that lack kinase activity[84,85]. Further mutagenesis studies of DNA-PKcs revealed that autophosphorylation of T946, S1004 and T3950 inactivates NHEJ and promotes homologous recombination. Therefore, DNA-PKcs autophosphorylation is likely to be a crucial mechanism for ensuring the appropriate use of homologous recombination. This idea is supported by the observation that mice with targeted substitution of multiple DNA-PKcs autophosphorylation sites die at a very early age and are defective in homologous recombination[86]. Altogether, these findings are consistent with a model in which NHEJ and homologous recombination factors are in active competition for the repair of DNA double-strand breaks. If NHEJ is not initially successful, displacement of NHEJ factors, perhaps by double-strand break resection, might enable error-free repair activities to dominate.

Factors favouring translocations

Experimentally induced double-strand breaks on different chromosomes are known to significantly increase the rate of translocation between those chromosomes[87]. Evidence in favour of a DNA double-strand break intermediate in translocation has come from the study of translocations between the immunoglobulin constant region genes *IGKC* and *IGLC1*, as well as *MYC* in Burkitt's lymphoma. First, double-strand breaks at the *IGH* locus are known to occur because of the action of the activation-induced cytidine deaminase (AID) enzyme, which deaminates target cytidine residues, leading to the appearance of staggered double-strand breaks[88]. In the absence of AID, translocations between these two genetic loci occur at vanishingly low frequency[89], demonstrating the importance of double-strand breaks as a substrate for translocation. Experimental systems using site-specific, inducible DNA double-strand breaks have also shown that translocation between two sites is highly dependent on the frequency of double-strand breaks[6,7]. Further evidence for a double-strand intermediate leading to translocation came from an analysis of translocation frequency in p53-knockout mice. One activity of the tumour suppressor gene *TP53* is

to promote apoptosis in cells with double-strand breaks, and deletion of p53 or upstream components of the DNA damage signalling pathway, such as ATM, increases the overall frequency of translocations[90]. This shows that providing an environment that is favourable to DNA double-strand breaks promotes translocation.

The appearance of recurrent translocations, such as *IGH–MYC* in Burkitt's lymphoma, has posed the question of why certain translocations occur so commonly in specific malignancies. One possibility is that these recurrent translocations arise at no more common a frequency than any other translocation but that they are selected on the basis of their potential to drive survival and proliferation of the cancer cell. An additional, long-standing hypothesis is that recurrent translocations arise because the translocation partners are in particularly close proximity to the nuclei of cells from the affected tissue[91–93]. Chromosome conformation capture has been used to measure genomic interactions, and combining this technique with deep sequencing has recently enabled the measurement at base-pair resolution of how closely genomic loci interact. Using this approach, Hakim *et al.*[5] showed that *IGH* and *MYC* do not interact particularly closely in activated B cells[5]. In fact, even though *IGH–MYC* translocations are found in 85% of Burkitt's lymphoma, 2,361 other genes interact with *IGH* more often than *MYC*. This suggests that nuclear proximity is not the key driver of recurrent, cancer-associated *IGH–MYC* translocations. Moreover, Rocha *et al.*[94] showed that there is a poor correlation between genes that physically interact with *IGH* and those that are AID targets[94]. The authors proposed that AID targets are situated in broader genomic domains that associate with *IGH*, but the current evidence seems to indicate that physical proximity in the nucleus is a minor determinant of translocation frequency between genes on different chromosomes.

The quantification of how frequently a double-strand break at a specific site forms translocations with other genomic loci has recently become feasible owing to the development of two similar techniques: high-throughput genome-wide translocation sequencing (HTGTS) and translocation-capture sequencing (TC-seq)[6,7]. These studies were carried out in B cells and focused again on understanding what factors determine the translocation partners of double-strand breaks at *IGH* or *MYC*. In both studies, there was a strikingly high correlation between AID target sites and translocation frequency. This suggests that genes that are more often affected by double-strand breaks form translocations more readily. Transcriptional status is another factor that influences translocation frequency, as most translocations were to coding sequences, and transcribed genes were more commonly subject to translocation than were silent genes. Translocation partners for double-strand breaks are not strictly limited to closely interacting chromosome domains, but when a large number of double-strand breaks is present, there is an increased frequency of interchromosomal translocation between partners with higher physical interaction[95]. All deep-sequencing studies to date have shown that double-strand breaks on the same chromosome, particularly those lying nearby on the same chromosome, have the highest rate of joining, and this matches a previous study of the frequency of joining of breaks induced by the V(D)J recombination-activating protein 1 (RAG1) and RAG2 recombinases[96].

Taking these studies together, the primary predictor for whether genes take part in translocations is the frequency with which those genes undergo double-strand breakage. Hence, translocations between *MYC* and *IGH* are favoured in B cells because those regions are common sites for double-strand breaks in B cells.

Table 1 | **Phenotypes of loss-of-function NHEJ mutations**

NHEJ gene	Mouse knockout phenotype	Patient phenotype
XRCC6 (encoding Ku70)	Viable, SCID, small size, radiosensitivity and thymoma[50,51]	None known
XRCC5 (encoding Ku80)	Viable, SCID, small size, radiosensitivity, genomic instability and tumours, especially with p53 deletion[47,52–54]	None known
PRKDC (encoding DNA-PKcs)	Viable, SCID, some genomic instability and tumours with p53 (REFS 55–57)	Human hypomorph has SCID and radiosensitivity[58]
DCLRE1C (encoding Artemis)	Viable, SCID, radiosensitivity and genomic instability[59]	Null results in SCID and radiosensitivity; hypomorph shows reduction in lymphocytes, genomic instability and lymphoma[60,61]
NHEJ1 (encoding XLF)	Mild lymphocytopaenia and radiosensitivity[62]	Cernunnos syndrome; immunodeficiency, developmental delay, microcephaly, reduced growth and genomic instability[63]
XRCC4	Null is lethal with neuronal apoptosis; rescue with p53 results in SCID, radiosensitivity, early B lymphoma and genomic instability[49,64]	None known
LIG4	Knockout is lethal with neuronal apoptosis; rescue with p53 results in pro-B lymphoma and radiosensitivity; hypomorph is small, lymphopaenic and has reduced haematopoietic stem cell function[65,66]	LIG4 syndrome; immunodeficiency, reduced growth, developmental issues, microcephaly and malignancy[67,68]

DCLRE1C, DNA cross-link repair 1C; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; LIG4, DNA ligase 4; NHEJ, non-homologous end-joining; NHEJ1, NHEJ factor 1; PRKDC, protein kinase, DNA-activated, catalytic polypeptide; SCID, severe combined immunodeficiency; XLF, XRCC4-like factor; XRCC, X-ray repair cross-complementing protein.

Active transcription also correlates with translocation, and up to 40% of translocations involve the joining of a break to a sequence from the same chromosome. These intrachromosomal translocations are not seen recurrently in cancer cells. This suggests that many translocations do not contribute to tumorigenesis and that oncogenic translocations that increase cell survival and proliferation are selected for during the evolution of a tumour.

As the frequency of double-strand breaks at a particular genomic site seems to be the key determinant of whether that site becomes involved in a translocation, it is important to understand the processes that produce double-strand breaks. DNA replication is a source of DNA double-strand breaks[97]: as the entire genome is replicated during cell division, any genomic site is a potential site for replication-associated double strand breaks. However, replication-associated DNA damage is not entirely random. Common fragile sites (CFSs) are regions of the genome that are prone to breakage during replication stress. Whereas CFSs are relatively stable in normal cells, cancer cells accumulate breaks and genomic aberrations, including translocations, at these sites[98,99]. Breakage at CFSs in cancer cells seems to be a consequence of replication stress arising from accelerated, oncogene-mediated replication[100–104]. In addition to CFSs, a second class of genomic sites that are prone to double-strand breakage and are associated with translocations in human cancer has recently been identified in murine B cells[105]. These early replicating fragile sites (ERFSs) were identified using ChIP followed by deep sequencing to reveal sites that are preferentially bound by DNA damage response proteins after replication stress. ERFSs are distinct from CFSs because they are found around early replication origins, whereas CFSs replicate late in S phase. ERFSs also have a high GC content, are commonly associated with repetitive DNA elements and correlate with transcriptionally active genes in an open chromatin environment. Breakage at ERFSs is AID independent, hence these sites may also be present in other cell types. Some euchromatic regions are targets of both AID activity in G1 and ERFS fork collapse during S phase; however, whereas AID activity is limited to 1–2 kb of promoters, breakages at ERFSs span a much larger region ranging from 10 kb to 1,000 kb[105].

Chromatin and transcriptional status are likely to play a substantial part in determining the likelihood that a particular genomic site will be involved in a translocation. A correlation of translocations with transcriptional activity was noted from deep-sequencing studies[6,7], and transcription has been reported to predispose genomic fragile sites to DNA breakage by causing increased

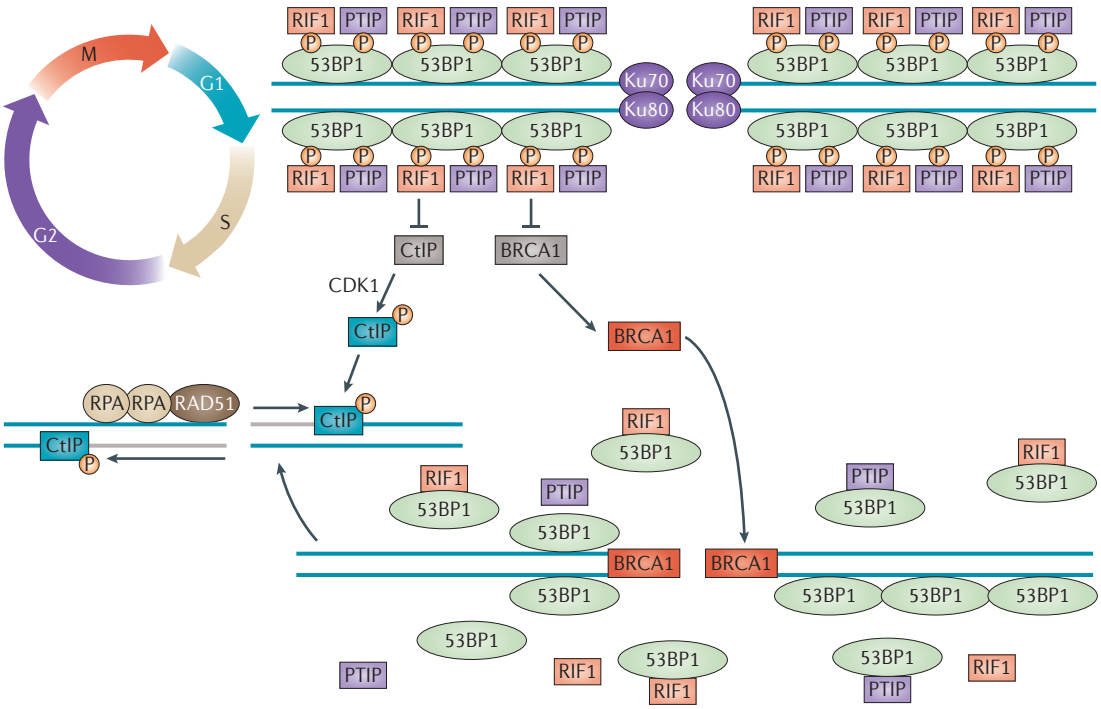

Figure 5 | **Regulation of DNA double-strand break repair pathways.** Non-homologous end-joining mediated by Ku70 and Ku80 is favoured in G1 phase, when the activities of BRCA1 and CtBP-interacting protein (CtIP) are repressed by a complex of p53-binding protein 1 (53BP1), Pax transactivation domain-interacting protein (PTIP) and RAP1-interacting factor 1 (RIF1), which coats the chromatin in the vicinity of double-strand breaks. During the transition to S/G2 phase, BRCA1 acquires the ability to bind at break sites, where it promotes loading of repair factors either by direct displacement of the 53BP1 complex or by remodelling of the chromatin environment at the DNA break. The mechanism for BRCA1 activation and recruitment is still unknown. Also during S/G2 phase activation by cyclin-dependent kinase 1 (CDK1)-mediated phosphorylation (P) allows CtIP to become active at the break site, where it resects duplex DNA to form a 5′ single-strand overhang. This favours resection-dependent repair pathways, including alternative end-joining and homologous recombination. Commitment to homologous recombination is mediated by loading of replication protein A (RPA) and RAD51 at the single-stranded DNA region formed by resection at the DNA break site.

collapse of DNA replication forks[106]. Although the existing data indicate that transcriptionally active regions are more prone to translocations, γH2AX, which signals DNA damage, accumulates more readily in euchromatic sites than in heterochromatin[107,108]. Double-strand breaks also take longer to repair when located in heterochromatin[109]. Heterochromatin protein 1α (HP1α; also known as CBX5) paralogues are recruited to break sites[110–112], and depletion of HP1α or of the nucleosome assembly complex component chromatin assembly factor 1 (CAF1) inhibits repair by homologous recombination[113].

Translational opportunities and perspectives

Sequencing-based approaches have enabled considerable progress in recent years in understanding the nature and effect of chromosome translocations. We now have a much clearer idea of the frequency and complexity of translocations. Although translocations are almost invariably found when we study the genomic landscape of cancer cells, the importance of translocations to the onset of malignancy is still a matter of debate. In contrast to the situation with the characteristic clonal translocations identified in CML and Burkitt's lymphoma, many translocations do not seem to be primary drivers of cancer cell growth. We are still at an early stage in analysing the sequencing data that are pouring in, and making sense of how translocations influence cancer cell growth will be a major topic of research interest in the coming years. Another major challenge lies in understanding the cellular pathways that underpin the genomic complexity of cancer cells. What pathways are responsible for causing translocations? Is there a role for replication-based mechanisms in the formation of translocations, and what is the importance of microhomology at translocation junctions? Answering these questions will require us to build on our current understanding of the genes involved in translocation pathways, enabling us to test requirements for the appearance of translocations *in vivo*. Sequencing is demonstrating that many translocations are more complex than we had previously imagined[65]. It will be interesting to see whether chromothrypsis[2] arises by different pathways to translocations or whether it merely represents the most extreme end of a range of chromosome rearrangements present in cancer cells.

Although we have made major progress in understanding nuclear phenomena that influence the frequency of translocations, there are clearly remaining issues to address relating to the effect of chromatin on genomic instability and translocation frequency. At the time of writing, there has been no published, genome-wide attempt to correlate chromatin status with translocation frequency. Such work would shed light on several interesting studies that have demonstrated how chromatin can affect the processing of DNA breaks. Future work is also likely to identify other factors in mammalian cells which, as is the case with 53BP1, PTIP, RIF1, Ku and DNA-PKcs, are able to modulate the use of NHEJ pathways. The activity of such factors could determine the frequency of translocations by biasing the repair of DNA breaks to error-prone end-joining pathways.

As translocations seem to be mainly produced by the C-NHEJ and A-EJ pathways, selective inhibition of end-joining pathways could potentially be used to prevent the appearance of cancer or to block the appearance of further mutations that drive cancer growth and survival. Global inhibition of end-joining is unlikely to be a beneficial long-term treatment modality on the basis of observations in gene-targeted mice that correlate loss of end-joining activity with increased chromosome abnormalities and tumour incidence. Nonetheless, cancer cells seem to make use of A-EJ pathways to join DNA double-strand breaks in aberrant ways that promote cancer growth[114]. Acquired resistance of BRCA2-deficient cells to poly(ADP-ribose) polymerase (PARP) inhibition has been shown to occur by A-EJ-mediated internal deletions within the *BRCA2* gene that restore its activity[115,116]. These observations reinforce the importance of understanding the genetic requirements of A-EJ to enable specific targeting of this pathway.

Inhibitors of DNA ligases have been identified and shown to be toxic towards cancer cell lines and to synergize with methyl methanesulphonate treatment to increase cell killing[117,118]. Inhibitors of DNA-PKcs have likewise shown promise, potentially on the basis of their ability to bias DNA repair towards homologous recombination instead of more toxic pathways[119]. Although animal studies are currently lacking, in the future agents that enable repair to be shifted from mutagenic pathways towards repair pathways that promote faithful DNA repair (as has been proposed for inhibition of 53BP1 in patients with BRCA1 deficiency[34]) could provide a new avenue for cancer treatment that is based on the prevention of mutation.

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Competing interests statement
The authors declare no competing financial interests.

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